

**UNIVERSITY OF DERBY**

**HUMAN EXPOSURE ASSESSMENT OF  
FLUORIDE FROM TEA (*CAMELLIA SINENSIS L.*)  
WITH SPECIFIC REFERENCE TO HUMAN  
BIOACCESSIBILITY STUDIES**

A thesis submitted in partial fulfilment of the requirements of  
the University of Derby for the degree of Doctor of Philosophy

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**Doctor of Philosophy**

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## **Declaration of originality and statement of copyright**

The work presented in this thesis is original and the work of the author. References to all non-original material have been cited in the text with full reference list provided following this work.

Signed                      Laura Chan

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*Dedicated to my children, Jade, Jamie and Luke*

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## Abstract

This study aims to determine the concentrations of fluoride in UK tea products and their infusions. This is related to the uptake and distribution of fluoride within tea plants *Camellia sinensis* (L.). Human oral bioaccessibility of fluoride from the consumption of tea infusions was estimated, using an *in vitro* approach. The possible health significance from fluoride exposure is discussed.

Fluoride in tea products and the distribution within the tea plant was determined using a method, involving alkali fused digestion with ion chromatography and a conductivity detector for the instrumentation. For the aqueous infusions and the supernatants in the bioaccessibility experiments, ion selective electrode with a voltmeter was adopted.

Mean fluoride concentrations in tea products and their infusions varied significantly ( $p < 0.001$ ;  $n=3$ ) and were related to the type of tea product and the retail cost. The higher priced teas, such as Darjeeling, Assam and Oolong, had lower fluoride concentrations. The lower priced supermarket Economy ranged teas were significantly higher ( $p < 0.05$ ) in fluoride and exhibited concentrations similar to Chinese Brick tea, which is prepared using mature tea leaves. The higher quality products are prepared by selecting the finest tips of tea (buds), whereas an Economy products use coarser harvesting techniques to include mature leaves in the product.

Fluoride affinity and tolerance of *C. sinensis* was assessed by a series of fluoride dosing experiments, ranging from 0 to 200 mg. Following fluoride dosing, a rapid uptake and accumulation occurred throughout the tea plants, resulting in partial necrosis of random leaves. Despite the necrosis, the plants tolerated the fluoride and continued to increase in height, although at a significantly slower rate ( $p < 0.05$ ) compared to the control plants. Accumulation of fluoride was observed to be mostly in the mature leaves followed by younger buds, then the roots. This relates to the part of the plant

used to produce the tea types, with mature leaves for Economy products and the buds for the finer teas.

The *in vitro* bioaccessibility assessment of fluoride estimated that over 91.4% of fluoride from a tea infusion is available in the human gastric compartment, with 92.1% in the gastro-intestinal compartment. The addition of milk reduced fluoride absorption in the gastric and gastro-intestinal compartments to 73.8 and 83.1%, respectively, possibly reacting to form calcium fluoride. Despite the percentage bioaccessibility, the concentration of fluoride available for absorption in the human gut was dependent upon choice of tea product. Based on an adult male, the findings suggest that consuming a litre of Economy tea can fulfil or exceed (75 to 120%) the recommended dietary reference intake (DRI) of fluoride at 4 mg a day, but only partially fulfil (25 to 40%) when consuming a more expensive Pure blend such as Assam.

With regards to health, tea consumption is a source of fluoride in the diet and is highly available for absorption in the human gut. Tea alone can fulfil an adult fluoride DRI, but is dependent upon choice of tea product. Excess fluoride in the diet can lead to detrimental health effects such as fluorosis of the teeth and skeletal fluorosis and consuming economy branded tea can lead to a higher exposure.

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## **ABBREVIATIONS**

ANOVA = Analysis of variance  
CFC = Chlorofluorohydrocarbon  
CRM = Certified reference material  
DNA = Deoxyribonucleic acid  
DRI = Daily reference intake  
GC-MS = Gas chromatography with mass spectrometry  
HF = Hydrofluoric acid  
IBM = International Business Machines  
IC = Ion chromatography  
ICP-AES = Inductively coupled plasma atomic emission spectroscopy  
ICP-MS = Inductively coupled plasma mass spectroscopy.  
ICP-OES = Inductively coupled plasma optical emission spectroscopy.  
IPGRI = International Plant Genetic Resources Institute (Now known as: Bioversity International)  
ISE = Ion selective electrode  
LGC = London Government Chemists  
NaF = Sodium fluoride  
NAS = National Academy of Science  
NIST = National Institute of Standards and Technology  
PBET = Physiologically based extraction tests  
PTFE = Polytetrafluoroethylene  
SPSS = Statistical Package for the Social Sciences (IBM Cooperation)  
SD = Standard deviation  
TMAH = Tetramethylammonium hydroxide  
USA = United States of America  
UTI = Upper tolerable intake



# **Chapter 1: Introduction**

## 1.1 The tea plant and beverage

### 1.1.1 Introduction

Tea is a member of the genus *Camellia* and the family of Camelliaceae (*Theaceae*). It is an evergreen perennial shrub with several varieties, but many different cultivars, including *C. sinensis* (L.) O. Kuntze, *C. assamica* ssp and *C. assamica* ssp. *lasiocalyx* (Banerjee 1992; Ahmend and Stepp 2013). It is a heterogeneous plant renowned for its ability to hybridise naturally, hence, the wide variety of types of teas (approximately 1,500) and tea products (Banerjee 1992).

Tea is one of the world's favourite drinks and therefore demand for the crop is high, and indeed cultivation has increased worldwide (Menon 2010). Although tea growing originated in China, plantations now exist across India, Sri Lanka, Indonesia, North and South America and certain European countries, including the UK (Wyke 2008; Tea Council 2013a). Most plantations exist in areas where the air temperature is humid between 20 - 25 °C, 600 - 2000 metres above sea level and in acidic soils with a pH of less than 5.6 (Carr and Stevens 1992). Apart from tea plantations, *C. sinensis* can be found growing naturally in forests in China, Japan, Thailand and South Korea (Williges 2004).

### 1.1.2 History

Tea consumption originated in China with the earliest known records dating to the 10<sup>th</sup> century BC (Crosby 2007). Around the 3<sup>rd</sup> century BC, tea was noted for its medicinal properties where the legendary Emperor Shennong of China, an inventor of Chinese medicine, was said to use tea as an antidote against certain 'poisons' (Evans 1992). Buddhist monks introduced tea and cultivation skills to Japan around 600 AD (Evans 1992). Much later, around the 17<sup>th</sup> century, tea arrived in Europe and

the USA from eastern shipping imports. Initially this was a beverage for the wealthy, but by the end of that century with increasing imports, tea was consumed at all levels of society around the world (Evans 1992).

Today, tea continues in popularity with production and consumption estimated at 3.6 million tonnes in 2006 (Antonios 2008). China is the leading manufacturer, producing approximately 1,640,310 tonnes of tea, followed by India with a 966,733 tonne production in 2011 (Faostat 2013a). As of 2009, Paraguay consumed the most tea annually (kg per capita), followed by Uruguay, then Argentina (Faostat 2013b). However, the Tea Council (2013b) state the Republic of Ireland followed by Britain as the highest consumers per capita in the world. Over 170 countries consume of one or more varieties of tea (Faostat 2013b). Tea popularity continues in many parts of the world, with approximately 165,000,000 cups consumed daily in the UK (Tea Council 2013b). The UK Tea Council (2013a) state an average of 3 cups of tea are consumed per person daily in the UK and recommend 4 cups of tea a day for optimal health benefits, which represents approximately 1000 ml a day. Significant health benefits include the level of fluid intake and the presence of antioxidants in the tea (Tea Council 2013a).

### **1.1.3 Varieties of tea beverages**

Different manufacturing processes characterise the type of product produced, such as black, green, white, yellow, Keemun or Oolong (Gascoyne *et al.* 2011). Young leaves and buds are hand-picked or machine harvested to produce the different tea beverages (Tea Council 2013b). Hand plucking has mostly been favoured, but this method is very labour intensive and costly, so many countries have adopted machinery especially in Japan and Argentina (Owuor *et al.* 2011). Machine harvested tea may include some mature leaves rather than hand selecting the top bud, also leaf

damage can occur, which can be reflected in the quality and taste of the product (Obanda and Owuor 1995; Ravichandran and Parthiban 1998).

Tea manufacturers have ascertained that different varieties, locations, and seasons tend to produce better qualities of certain classes of tea (Carr and Stevens 1992). For instance, the same genotype of tea plant may be imported and grown at a lower altitude in a new location and this tea will lack aromatic qualities compared to those grown in high altitude (Owuor *et al.* 2011). Climate can also affect the crop yield and quality with preference for warm temperatures with moist, acidic soils (Fung *et al.* 2003; Li *et al.* 2007; Owuor *et al.* 2011).

The most common tea blend products are:

**Black blends** are teas that have undergone the full oxidation process and usually consist of blends of teas from many different countries (Tea Council 20013b). Economy blends are also black blended teas, but labelled as essential economy brands by UK supermarket chains. These products often cost much less than the branded market leaders. Assam and Darjeeling are classified as black teas, but are from a specific geographical location, Northeast India. In certain regions of the Eastern world, such as Tibet and China, brick tea is consumed, prepared like black tea but using mature leaves and tea branches (Chao *et al.* 1995).

**Green blends** are teas that are prevented from oxidising by the application of direct heat in roasting pans (Gascoyne *et al.* 2011). The leaves are shaped by hand or machine rolling and the final product retains the natural green colour, hence the name (Gascoyne *et al.* 2011).

**Oolong tea** leaves are normally rolled and undergo a partial oxidation process, followed by heating in a roasting pan to prevent further fermentation (Gascoyne *et*



*al.* 2011). The degree of oxidation can vary depending upon the type of Oolong required, for example, at 20% oxidation the product will form a green Oolong (Pettigrew and Richards 2008). A 60% oxidation produces a darker tea, known as Formosa Oolong (Pettigrew and Richards 2008).

**Pu'er tea** is a speciality tea made from sun dried green leaves (Gascoyne *et al.* 2011). The tea is then fermented in a process similar to composting (Gascoyne *et al.* 2011). Pu'er teas are sold in a loose leaf form or compressed into a brick form; both forms have long shelf lives compared to other varieties of teas (Pettigrew and Richards 2008).

#### **1.1.4 Chemical composition**

The shoots used to manufacture the beverage contain complex chemical compounds, including proteins, organic acids, polyphenols, caffeine, carbohydrates, minerals, fibre and vitamins (Kumar *et al.* 2013). Polyphenol compounds have received much attention, especially relating to perceived health benefits (Imai *et al.* 1997; Shirakami *et al.* 2012; McQuade *et al.* 2013; Sanchez-Tena *et al.* 2013). Catechins are included in the polyphenol group, such as (-)-epicatechin (EC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin (EGC), and these account for approximately 25% of the dry weight of unprocessed tea (Kumar *et al.* 2013). Vitamins in tea include A, B, C, E and K; with minerals of aluminium, iron, copper, fluoride, manganese and zinc also present (Mehra and Baker 2007; Kumar *et al.* 2013).

#### **1.1.5 Human health and nutrition from tea**

Tea has many health benefits, for example, tea polysaccharides have been reported to have bioactivities, including anti-oxidant, anti-blood coagulant, anti-HIV and anti-

radiation properties (Chen and Xie 2001). Extensive research on health promoting effects of green tea has been carried out recently. Effects include anti-cancer, anti-diabetic and anti-obesity activities (Schneider and Segre 2009; Suzuki *et al.* 2012). This has lead to an increase in green tea products and sales in certain countries (Lee and Chambers 2007; Mintel 2012). In Britain, since 2011, green tea sales increased by 83% with approximately 12% of Britons now consumers (Poulter 2012).

An epidemiological study of 8500 people in Japan, over a nine year period, concluded that regular consumption of green tea gave a potentially preventive effect against all sites of cancer, especially in those having the highest consumption (Imai *et al.* 1997). A cohort study was conducted by Setiawan *et al.* (2001) in Yangzhong, China, on 133 stomach cancer cases, 166 chronic gastritis cases, and 433 healthy controls. Green tea drinking was found to be protective against chronic gastritis and stomach cancer. In addition, dose-response relationships were observed with years of green tea drinking in both diseases (Setiawan *et al.* 2001). In Birmingham, UK, a study showed that average fat oxidation rates were 17% higher after ingestion of green tea extract than after ingestion of a placebo. In addition, the study suggests green tea can also improve insulin sensitivity and glucose tolerance in healthy young men (Venables *et al.* 2008). Gardner *et al.* (2007) reviewed the benefits from black tea consumption. An intake of at least three cups a day was suggested to prevent chronic heart disease and one to six cups a day increased plasma antioxidant capacity (Gardner *et al.* 2007). Tea consumption can account for up to 100% of the daily dietary intake of the essential mineral, manganese (Mehra and Baker 2007).

In contrast to the beneficial health effects of tea, adverse effects have also been reported. Tea can lead to the inhibition of iron absorption and anaemia in humans, especially if receiving a vegetarian diet, although this may be beneficial for individuals suffering with excessive iron storage diseases (Samman *et al.* 2001; Reddy *et al.* 2006). According to Jain *et al.* (2013), the phenolic antioxidant compounds

perceived to be beneficial for health can act as pro-oxidants, forming reactive oxygen species which can damage DNA and biological molecules. Polyphenols in green tea can affect the bioavailability of drug absorption by binding to the drug molecules (Yang and Pan 2012), so it may not be advisable for people relying on medication to consume green tea. Tea catechins are also reported to limit enzyme related drug transportation and metabolism (Feng 2007).

## 1.2 Fluoride

### 1.2.1 Chemistry

Fluorine is one of the halogen elements from group VII of the periodic table. It is the most reactive element known, due its strong electronegative behaviour. With its small atomic size, a strong pulling force holds the 7 electrons in the outer shell tightly to the nucleus, forming a -1 oxidation state (Atkins *et al.* 2010). Therefore, it does not normally exist in its natural elemental state of fluorine, but as salts of the monovalent halide ion, fluoride ( $F^-$ ). It will bond with practically every known substance even with one of the most un-reactive elements, such as xenon from the noble gas group VIII (Wieberg *et al.* 2001; Atkins *et al.* 2010). In isolation, fluorine can exist in its diatomic form ( $F_2$ ) where it is an extremely toxic pale yellow gas. However, the F-F bond is weak due to electron-electron repulsion of the ion pairs and this also aids its ability to form compounds. With the -1 oxidation state, fluoride is a strong oxidising agent and Lewis acid, forming high oxidation states, for example, antimony pentafluoride ( $SbF_5$ ), sulphur hexafluoride ( $SF_6$ ) and iodine heptafluoride ( $IF_7$ ) (Wieberg *et al.* 2001). Fluoride will also react with metals to form the metal halide, such as sodium fluoride (NaF) and calcium fluoride ( $CaF_2$ ).

Organic fluoride compounds form very strong chemical bonds, and hence have many uses (Wieberg *et al.* 2001). For example, the polymer polytetrafluoroethylene (PTFE)

is chemically and thermally stable; used for non-stick cooking equipment and thread seal tape (Earl and Wilford 2000). Chlorofluorohydrocarbons (CFC's) were widely used in aerosol spray cans and as refrigerant insulators. These are now banned from use due to a reaction involving chloride free radicals which destroyed the ozone layer (Tressaud 2006). However, fluoride was not directly involved in the formation of the hole in the ozone layer, as it reacts with water to form aqueous hydrogen fluoride or hydrofluoric acid (HF) (Harris 2010).

### **1.2.2 Fluoride in the environment**

The Earth's crust contains about  $0.3 \text{ gkg}^{-1}$  of fluoride, where fluoroapatite ( $\text{Ca}_5(\text{PO}_4)_3\text{F}$ ) is the most abundant mineral and calcium fluorite ( $\text{CaF}_2$ ) is the most common mineral extracted for industrial purposes (Fuge and Andrews 1988; WHO 2004). Volcanic activity and natural weathering processes of rock are natural sources of introducing fluoride into the environment. Mining activities, aluminium smelters, combustion of coal, applications of fertilisers, increased industrial use and fluoridation of water supplies are the main anthropogenic sources of fluoride (Arnesen 1997; WHO 2004).

With advanced technology, the use of fluoride in today's society has escalated, resulting in a demand for fluoride containing minerals (Edmunds and Smedley 2013). In addition, this has lead to an abundance of fluoride cycling through the environmental compartments (Edmunds and Smedley 2013). Fluoride can be introduced into the environment from many sources and its biogeochemical cycling is shown in Figure 1.1.

#### ***Air***

In the atmosphere, gaseous forms include hydrogen fluoride (HF), silicon tetrafluoride ( $\text{SiF}_4$ ) or hexafluorosilicic acid ( $\text{H}_2\text{F}_6\text{Si}$ ), with 75% of global airborne

fluoride existing as HF (International Programme on Chemical Safety 2002). Atmospheric particulate forms include sodium aluminium fluoride or cryolite, ( $\text{Na}_3\text{AlF}_6$ ), aluminium fluoride, calcium fluoride and calcium phosphate fluoride or fluorapatite ( $\text{Ca}_5(\text{PO}_4)_3\text{F}$ ) (International Programme on Chemical Safety 2002).

Anthropogenic fluoride usually occurs close to the emission source, whereas natural airborne sources from volcanoes or geysers can travel further emitting between 60 to 6000 Gg of  $\text{F}^-$  per year (Franzaring *et al.* 2007). Natural atmospheric fluoride background levels are usually less than  $0.1 \text{ } \mu\text{g m}^{-3}$  (International Programme on Chemical Safety 2002). However, this can increase in the proximity of industrial sources. For example, in the Netherlands close to industrial emissions, concentrations were reported to range from 0.3 to  $0.4 \text{ } \mu\text{g m}^{-3}$  (Sloof *et al.* 1989).

Hydrofluoric acid fog or aerosols can occur from water vapour, allowing transportation of the fluoride (International Programme on Chemical Safety 2002). As fog, it can remain close to the ground due to being denser than air, however, if the air temperature rises, the fog can lift (Schotte 1987). Wet deposition of particulate fluoride onto soil and surface waters may occur through rainout processes, which is the fate of many anthropogenic sources where a plume is situated in the clouds (Sloof *et al.* 1989). Dry deposition tends to occur close to the source, for example Sidhu (1982) found the rate of deposition on to soil, decreased significantly with increasing distance from a fluoride emitting stack.

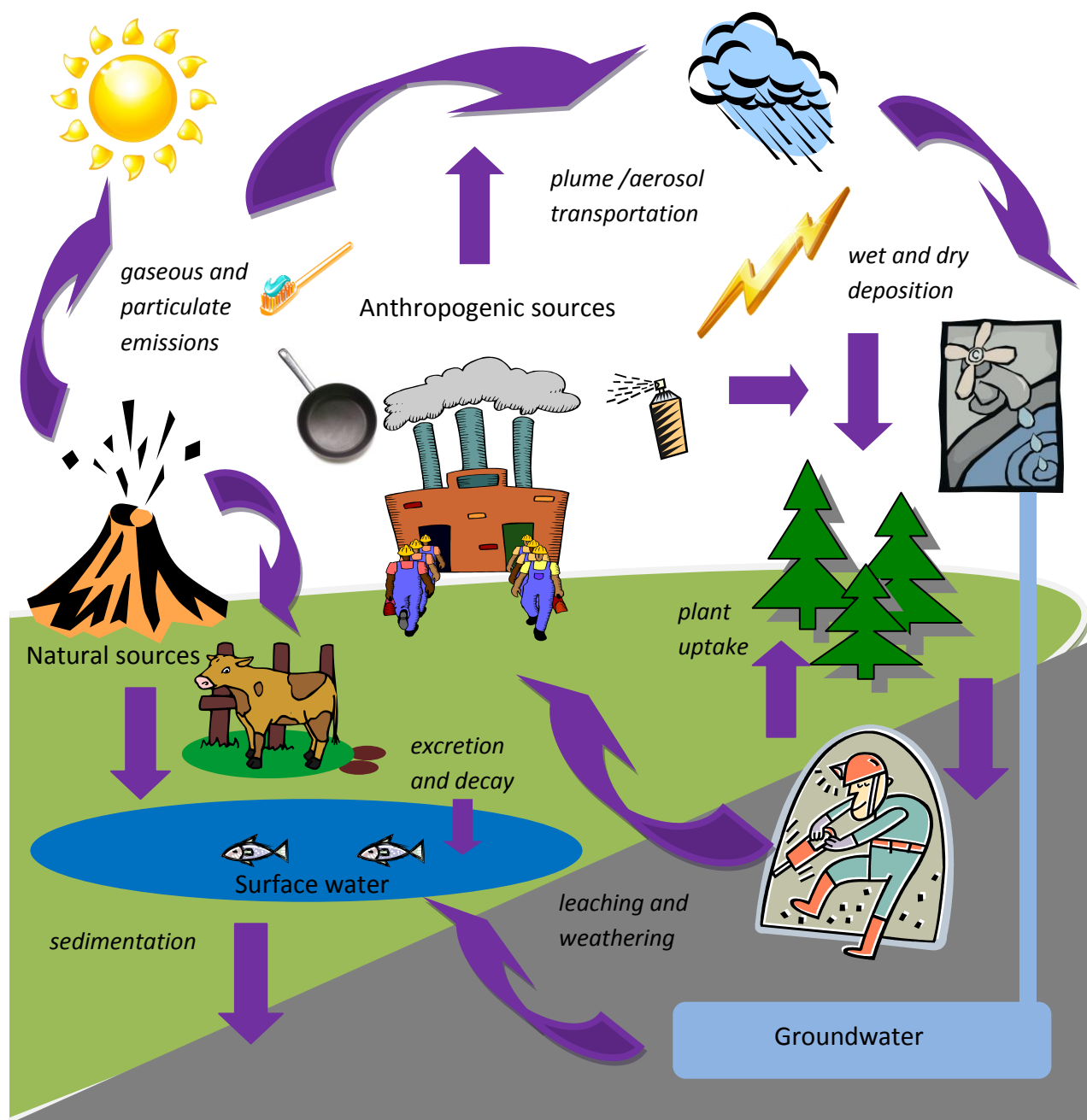


Figure 1.1 Cycling of fluoride in the environment from natural and anthropogenic sources.

### Water

Generally, background fluoride concentrations range from  $0.01$  to  $0.3 \text{ mg l}^{-1}$  and  $1.2$  to  $1.5 \text{ mg l}^{-1}$  in surface waters and sea water, respectively (International Programme on Chemical Safety 2002). Some groundwater can contain elevated concentrations

of fluoride due to the chemistry of the under lying rock. India is particularly affected, with concentrations of up to 20.6, 32.5 and 40 mg l<sup>-1</sup>, reported in Karbi Anglong, Delhi and Gujarat, respectively (Chakraborti *et al.* 2000; Raju *et al.* 2009). The highest fluoride containing surface water documented is the East Africa Rift Valley, where 2800 mg l<sup>-1</sup> was reported in Nakura Lake, Kenya, due to volcanic activity and hot climatic factors (Nair *et al.* 1984).

Transportation and release of fluoride from other environmental media into water depends upon pH, existing clays and the hardness of the water. In neutral waters, fluoride exists as the anion F<sup>-</sup>, with magnesium fluoride complexes being the next prevalent form (Edmunds and Smedley 2013). In acidic waters of less than pH 5.5 further fluoride can leach out of minerals, often transported through water as aluminium-fluoride complexes, such as AlF<sup>2+</sup>, AlF<sub>2</sub><sup>+</sup> and AlF<sub>3</sub>, if aluminium is present (Ares 1990). Sedimentation may occur with insoluble fluorides and soluble fluorides being vaporised or transported into the atmosphere as aerosols (Weinstein and Davison 2004).

### **Soil and rocks**

Fluoride concentration in rock varies depending upon rock type, for instance, ultramafic rocks and limestone can have concentrations of approximately 100 mg kg<sup>-1</sup> and marine shale's can contain around 1,300 mg kg<sup>-1</sup> (Faure 1991). The most common fluoride bearing minerals are found in certain types of ultramafic rocks (Table 1.1). Fluoride in soil is usually derived from the underlying parent rock, although some may be derived from volcanic ash. For example, when Mount Hekla erupted in Iceland in 1970, soil fluoride concentrations in the vicinity were recorded as high as 2000 mg kg<sup>-1</sup> (Bell 1998).

Weathering of parent rocks can release fluoride minerals, such as cryolite ( $\text{Na}_3\text{AlF}_6$ ), where it is broken down to release fluoride, especially under acidic conditions (Fuge and Andrews 1988).

Table 1.1 Fluoride minerals

**Content removed for copyright reasons**

Table data was extracted from:

Rao N. C. R. (2003). Fluoride and environment: A review. In: Bunch M. J., Suresh V. M. and Kumuran T. V.(Eds.), *Proceedings of the Third International Conference on Environment and Health, Chennai, India, 15-17th December 2003*. York University. pp. 386-399.

Generally, fluoride in soil ranges from 20 to 500  $\text{mgkg}^{-1}$  (Edmunds and Smedley 2013), although up to 1003  $\text{mgkg}^{-1}$  was reported in Canada as a fluoride reference soil sample (Schuppli 1985). For a contaminated petrochemical waste site in Oklahoma, USA, Schroder *et al* (1999) recorded a mean soil fluoride concentration of 1954  $\text{mgkg}^{-1}$ . Additionally, a study of total fluoride in background soils of the Peak District, UK, gave results ranging from 480 to 4450  $\text{mgkg}^{-1}$ , although the area is renowned to be rich in natural  $\text{CaF}_2$  (Saikat 2004). Noteworthy, water soluble fluoride is not available in these concentrations as fluoride in soil is strongly bound in complex compounds (Kabata-Pendias 2010).

The soils sorption capacity determines the fate and mobility of fluoride, where it is tightly adsorbed to clays and oxyhydroxides (Cronin *et al.* 2000). With minimal fluoride mobility occurring at pH 6.0 to 6.5, as the pH of soil increases to alkalinity, adsorbed  $\text{F}^-$  can be released and replaced by hydroxide ions ( $\text{OH}^-$ ) (Wenzel and Blum 1992). In acidic soils, of less than pH 6, adsorption to the soil is prevented by the formation of aluminium-fluoride complexes,  $\text{AlF}^{2+}$ ,  $\text{AlF}_2^+$  (Wenzel and Blum 1992).



Other factors affecting the mobility of fluoride from soil include phosphate from fertilisers, more so from top soils to lower soil horizons (Kabata-Pendias 2010). It is the water soluble fraction of fluoride that is of importance for the uptake by plants, so if it is not mobile, the cycling to other environmental compartments will not readily occur (Davison 1983).

### **Biota**

Aquatic plants and animals can accumulate fluoride from water, especially invertebrates and fish where the excess is stored in the exoskeleton and bone, respectively (Buse 1986; Gikunju 1992; Carmago 2003). Concentrations in crab (*Portunus depurator*) and prawn (*Crangon vulgaris*) were greater than 5 fold of the fluoride in the water with krill (*Euphausia crystallorophias*) exoskeletons containing concentrations of fluoride up to  $5477 \text{ mg kg}^{-1}$  (Sands *et al.* 1998; Carmago 2003). Bioaccumulation may be occurring through the food chain as Mikaelian *et al.* (1999) found mean bone fluoride concentration of whales (*Delphinapterus leucas*) to be  $4539 \text{ mg kg}^{-1}$  in a Canadian estuary.

In terrestrial biota, fluoride accumulation in mammals is quite extensive with lichens often used as a fluoride biomonitors after a volcano eruption or from an anthropogenic airborne source (Davies and Notcutt 1989; D'Alessandro *et al.* 2012). Soil fluoride is poorly available to plants being insoluble and adsorbed onto soil, especially when in the form of  $\text{CaF}_2$  (Cooke *et al.* 1976); however, some plants can accumulate fluoride, such as the Shaggy Pea (*Oxylobium*), Camellia's (*Camellia*) and Thorntree (*Acacia*), without any signs of toxicity (Weinstein and Davison 2004). Other species cannot tolerate fluoride, and become necrotic at low concentrations, such as the Gladiolus (*Gladiolus* sp.), although literature only reports the effects of airborne sources (Yan *et al.* 2010).

Cooke *et al.* (1978) examined fluoride uptake and translocation of fluoride in Sunflower (*Helianthus annus L.*) by growing plants in sand cultures under laboratory conditions. Factors such as pH, calcium and aluminium content, clay minerals and phosphate affected fluoride uptake (Cooke *et al.* 1978). This work was extended by Wenzel and Blum (1992) who highlighted that the mobility of fluoride is dependent upon soil pH and different plant species take up different amounts of fluoride from contaminated soils (Arnesen 2006). However, the relationship between fluoride in soil and its uptake by plants is not clear. Israel (1974) found a correlation between soil-fluoride concentration and its uptake by plants, whereas Geeson *et al.* (1998) did not find any such relationship. Saikat (2004) showed that in the fluorspar ( $\text{CaF}_2$ ) mining area of the Peak District, UK, Dock (*Rumex sp.*) accumulates high fluoride concentrations, but suggested a correlation did not exist between plant uptake and fluoride concentrations in the soil. In the form of insoluble  $\text{CaF}_2$ , fluoride bioavailability may be affected by being tightly bound to the soil (Cooke *et al.* 1976a).

### **1.2.3 Fluoride in human health**

Fluoride is an essential micro-nutrient for human health. Deficiency  $<1 \text{ mgkg}^{-1}$  can cause dental caries and in excess  $>2 \text{ mgkg}^{-1}$  can lead to dental fluorosis and skeletal fluorosis (MRC 2002; WHO 2004). Studies were carried out in Southern Asia - China, India and Tibet, where increased incidents of skeletal fluorosis and dental fluorosis were seen, as a result of excessive fluoride intake (Cao *et al.* 1998; Cao *et al.* 2005; Baskaradoss *et al.* 2008). In India, it is estimated that 17 States are affected with endemic fluorosis, including Andhra Pradesh, Gujarat and Rajasthan (Chinoy *et al.* 1992; Sunitha 2011). Plate 1.1 shows two children suffering with skeletal fluorosis from the villages of Nalgonda, Andhra Pradesh, where the source of fluoride is drinking water from naturally contaminated water wells (Sunitha 2011).



Plate 1.1 Children with skeletal fluorosis by India Waterportal

Available under a Creative Commons Attribution Licence 2.0 at  
<https://www.flickr.com/photos/indiawaterportal/8070412879/in/set-72157631729070999>

Since 1945, fluoride has been added to many water supplies, mostly in developed countries, with the intention of preventing dental caries and for healthy bone growth (Jones *et al.* 2005; Bryson 2006; Fordyce *et al.* 2007). However, there is much public debate worldwide over the benefits of fluoridating water, as excess fluoride is toxic and detrimental to health (Cohen and Locker 2001). The World Health Organisation (WHO), supported by the EU Scientific Committee for Food, recommends a guideline value of  $1.5 \text{ mg l}^{-1}$  of fluoride in drinking water, but this does not consider human exposure to fluoride from other sources (Europa 1996; WHO 2004; Fordyce *et al.* 2007). Alternative exposure includes ingestion from use of oral hygiene products, food and beverages especially if water is used to prepare these, and inhalation (Bo *et al.* 2003).

Many developed countries have stopped fluoridating water supplies, for example the Netherlands who fluoridated from 1960 to 1973, decided they had no legal basis to

allow the addition of chemicals to drinking water (Damen *et al.* 2005). Canada has reduced its fluoridation in certain States, including Calgary in 2011 (CBC 2011).

In the Republic of Ireland, 71% of water supplies are still artificially fluoridated compared to just 10 % in the UK, whereas Northern Ireland does not fluoridate at all (British Fluoridation Society 2013a). Water fluoridation is not the people's choice decision, but it is government controlled (Jones *et al.* 2005; Bryson 2006; Mansfield 2010; Awofeso 2012). Other schemes are available to provide fluoride in the diet, such as salt or milk fluoridation (Pollick 2013). Fluoridated table salt supplies are offered as an alternative in countries such as the Czech Republic, Germany and Switzerland, providing people with a choice (EU Salt 2010). Although in Jamaica, all salt products available are artificially fluoridated, limiting the choice of the consumer (Jones *et al.* 2005; Limeback and Robinson 2012).

#### **1.2.4 Fluoride in tea**

Tea plants can accumulate high concentrations of fluoride even when grown on uncontaminated soils, despite fluoride being a non-essential element to plants (Ruan and Wong 2001; Ruan *et al.* 2004; Weinstein and Davison, 2004; Kabata-Pendias 2010). Total fluoride concentration in tea leaves can vary between 8 and 1530 mgkg<sup>-1</sup> depending upon the hybrid variety (Ruan and Wong 2001; Weinstein and Davison 2004) and from 15 to 2965 mgkg<sup>-1</sup> depending upon location within the plant, Table 1.2 (Shu *et al.* 2003). An abundance of fluoride is stored in the mature leaves, with the higher concentrations found in the mature leaves compared to the younger leaves and buds. The leaves are generally known to be used as the main raw materials for the production of the dried tea beverage (Moseti *et al.* 2013).

Table 1.2 The Distribution of fluoride ( $\text{mgkg}^{-1}$ ) in different parts of tea plants from China

**Content removed for copyright reasons**

Table data was extracted from:

Shu W. S., Zhang Z. Q., Lan C. Y. and Wong M. H. (2003). Fluoride and aluminium concentrations of tea plants and tea products from Sichan Province, PR China. *Chemosphere*. **52** 1475-1482.

As the plant is the raw material, a large variability in the concentrations of fluoride present in dry weight samples of tea products from around the world is identified by Yi and Cao (2008). Tea leaf total fluoride concentrations can range from 2.1 to 1175.0  $\text{mgkg}^{-1}$ , 0.5 to 631.0  $\text{mg l}^{-1}$  for instant teas and 0 to 33.4  $\text{mg l}^{-1}$  in the tea beverage itself (Yi and Cao 2008). This could suggest that different parts of the plant may be used to produce different tea products, as the quality of tea declines with increasing fluoride concentration according to Lu *et al.* (2004). Therefore, mature leaves that contain higher fluoride concentrations could alter the quality of the tea.

### **1.2.5 Human exposure to fluoride from tea**

The tea beverage is reported to contain fluoride in varying concentrations, leading to potential exposure of fluoride to the human system. This can be estimated by determining the rate of transfer of fluoride into the tea infusion (Mehra *et al.* 2013). Although this is not necessarily the bioavailability of fluoride in tea as defined by the Environment Agency (2002), 'the fraction of the chemical that can be absorbed

through the gastro-intestinal system, pulmonary system and the skin', it can still be used as an indication for exposure. 'Available' fluoride can simply be evaluated as the amount available to enter a human receptor (Loehr 1999).

Many factors can affect the level of exposure, including the choice of tea brand, the amount of tea used to prepare an infusion, daily consumption and infusion time, which are all dependent upon the consumer (Sofuoglu and Kavcar 2008). In Britain, 98% favour tea bags and 96% add milk to a black tea brew (Tea Council 2013c). These variables all relate to the extent of possible human exposure to fluoride from tea.

Nutrition and health are important areas in research, to support dietary recommendations and food legislation (Zicari *et al.* 2007; Ten Torrella *et al.* 2011; McCarthy 2012; Patel *et al.* 2012). In addition, there is a growing public awareness of beneficial and adverse effects from food additives, whether naturally present or used as additives (Nocella and Kennedy 2012; Buttriss 2013). However, there is a lack of consensus in worldwide scientific testing when it comes to determining nutrients or compounds in food (Fernandez-Garcia *et al.* 2009). With the variation of food types and different food matrices, standardised methodology can be problematic. Initially, most studies to identify concentrations of nutrients or compounds in relation to health, were based on bioavailability testing, a term derived from biological availability (Saikat 2004; Schumann *et al.* 1997). Bioavailability is used to determine whether or not the concentration of a chemical in a matrix will affect an organism (Peinberg and Jager 2003). Over the last few decades, bioaccessibility of components in relation to health have become a fast growing area of research interest (Dean 2006; Fernandez-Garcia *et al.* 2009).

### 1.3 Aim and objectives

The aim of this project is:

To investigate the concentrations of fluoride in tea, *Camellia sinensis* (L.) with special reference to human bioaccessibility studies and to consider possible health related issues.

In order to achieve this aim, the objectives of the project are addressed as:

1. To develop analytical methods to determine total fluoride in tea products, infusions, plants and soil (Chapter 2).
2. To determine total fluoride in a range of mainly UK available tea products (Chapter 3).
3. To determine fluoride concentration in tea infusions prepared from the tea products, using a range of infusion times (Chapter 4).
4. To develop an *in vitro* method to estimate the human bioaccessibility of fluoride from tea consumption (Chapter 5).
5. To assess the uptake of fluoride from soil by the tea plant (*C. sinensis*) and its influence on the plants growth (Chapter 6).

## **Chapter 2: Development of methods to determine fluoride content in tea products, plants and soils**



## 2.1 Introduction

As a basis for the current study, practical and reproducible methods were required for the analysis of total fluoride in tea products, tea infusions, tea plants (leaves, stems and roots) and soil. Total analyte determination of a solid material, such as vegetation and soil, requires break down of the sample matrix using a suitable digestion (Dean 2003). Digestion is necessary to break all ionic and covalent bonds involved in complexing compounds within the matrix (Boruvka and Vacha 2006). This can be achieved by traditional techniques such as acid or alkali wet digestions or dry ash digestion; or by more recent techniques, such as microwave or pressurised bomb digestions (Malde *et al.* 1997; Grobler and Louw 1998; Polkowska-Motrenko *et al.* 2000; Huang *et al.* 2004; Tuzen *et al.* 2004; Boruvka and Vacha 2006). Once digestion is complete, a suitable instrumental analysis technique for the analyte of interest, in this case fluoride, is required. Choice of digestion method should consider the sample matrix, the analyte of interest, equipment and instrumentation available, method of analysis and health and safety concerns (Riley and Rosanske 1996).

Complete digestion of a matrix can only be achieved by using hydrofluoric acid (Boruvka and Vacha 2006). This acid will break down all materials, including silicate bonds and will allow for 100% of the total analyte to be determined (Boruvka and Vacha 2006). Use of hydrofluoric acid requires special training and is not widely favoured due to its extreme corrosive property and associated health and safety concerns (Hydrofluoric acid 2004). However, the analyte of interest in this study is fluoride, so hydrofluoric acid would not be suitable. Through the use of other acids, such as 'aqua-regia', perchloric and sulphuric acids, 100% break down is not achieved, but will provide what is known as the 'pseudo' total analyte, and for most analyses, this is sufficient (Boruvka and Vacha 2006).

Alkali digestion can be specific to the analyte of interest, for example the EPA (1996), Method 3060a describes the use of sodium hydroxide solution to digest samples

when determining hexavalent chromium Cr(VI). Other literature describes the use of alkaline fused digestion for the determination of fluoride, where sodium hydroxide solution is fused to the sample and this fusion is ashed in a furnace (McQuaker and Gurney 1977; Sparks *et al.* 1996; Malde *et al.* 1997). Alkali fusion can overcome problems other digestions may have in breaking down all the fluoride bound silicate complexes (Cooke *et al.* 1976).

Dry ash digestion is suitable for many sample types and is a cost efficient technique that does not involve any chemicals (Caper and Szefer 2012). The sample is heated in a furnace until digestion is achieved, resulting in ash. The volatility of the analyte must be considered if using this type of digestion as a loss of total concentration may result. For fluoride determinations using the dry ash technique could lead to the loss of volatile fluorides (Cooke *et al.* 1976).

The use of microwave assisted acid digestion is a technique carried out in pressurised sealed bombs (Kebbekus 2003). Microwave digestion is very aggressive, which reduces the reaction time required for the sample to breakdown, thus decreasing overall digestion time (Milestone 2011). Since the process takes place in sealed bombs, the chances of contamination and loss of fluoride are reduced (Grobler and Louw 1998). Grobler and Louw (1998) reported a 98.1% fluoride recovery of standard di-sodium fluorophosphate indicating the procedure is capable of breaking down fluoride covalent bonding.

Suitable detection methods for analysing fluoride concentration can be achieved using a range of instrumentation, such as gas chromatography with mass spectrometry (GC-MS), inductively coupled plasma atomic emission spectroscopy (ICP-AES) and ion chromatography (IC) with a conductivity detector (Ibe *et al.* 1999; Saha and Kundu 2003; Kage *et al.* 2008). Published literature generally favours the use of the ion selective electrode (ISE), where a fluoride selective electrode is used in

conjunction with a silver chloride reference electrode (McQuaker and Gurney 1977; Duckworth and Duckworth 1978; Jenkins 1991; Gulati *et al.* 1993; Chan and Koh 1996; Fung *et al.* 1999; Kalayci and Somer 2003; Malinowska *et al.* 2008; Li *et al.* 2009). ISE's consist of the ion-selective membrane, an internal reference electrode, an external reference electrode and a voltmeter (Figure 2.1). Electrical potential of the fluoride ion (mV) is measured; in accordance with the Nernst equation, dependent on the logarithm of ionic activity (Rundle 2013). The calibration is plotted on a logarithmic scale and the sample results are anti-logged, taking the weight into consideration to give the fluoride concentration in  $\text{mg l}^{-1}$ . The limit of detection for fluoride determination is  $<0.1 \text{ mg l}^{-1}$  (Rundle 2013).

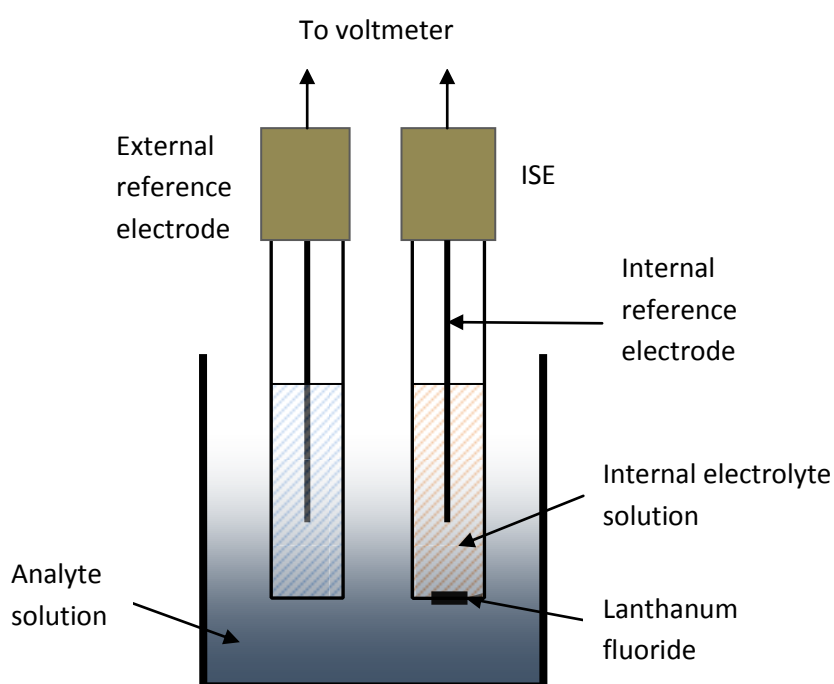


Figure 2.1 Schematic of an Ion Selective Electrode (ISE) measurement

IC is a form of liquid chromatography. Ionic species are measured by separation through interactions within a resin, dependent upon size and type of the species

(Haddad and Jackson 1990). A known aliquot of liquid sample is introduced into a pressurised chromatographic column where ions are absorbed by column constituents. The eluent, an ion extraction liquid, flows continuously through the column, at a specific flow rate and the absorbed ions separate from the column. A chromatogram is produced as a series of peaks, see Figure 2.2. Each peak represents a specific analyte, identified by retention time and the peak area determines the ionic concentrations in the sample (Haddad and Jackson 1990). Limit of detection using modern equipment can be as low as single figured  $\mu\text{g l}^{-1}$  for anions and cations (Bruce 2012).

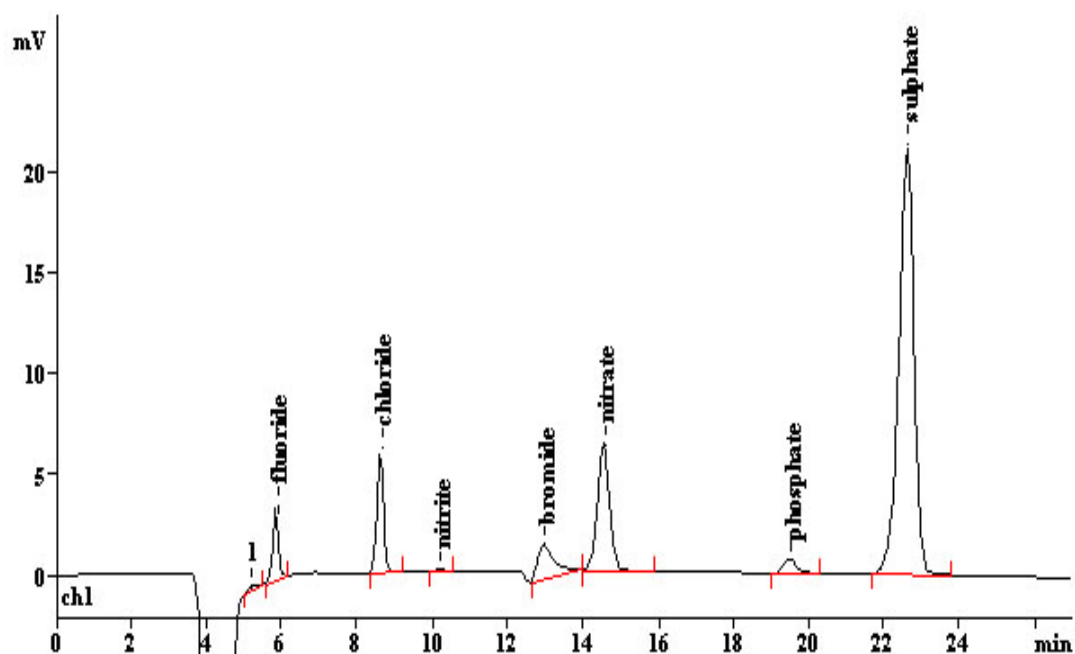


Figure 2.2 IC chromatogram showing anionic separation

IC can be more expensive, as the equipment needs regular servicing and replacement of parts, therefore more costly than ISE (Gossman 2007). Gossman (2007) suggests ISE is less reliable and not as accurate as IC, after conducting an inter-laboratory validation test determining the concentration of nitrates, but fluorides were not examined.

Total fluoride in tea infusions involves the extraction of water soluble fluoride from the tea beverage. This is achieved by adding boiling water to a known amount of tea leaf product and allowing a set infusion time. Infusion is stopped by removing an aliquot of liquor or by filtering (Fung *et al.* 1999; Chandrajith *et al.* 2007). The cooled supernatant can then be analysed for fluoride concentration. Numerous studies exist adopting the ISE technique for detection (Smid and Kruger 1985; Schamschula *et al.* 1988; Chan and Koh 1996; Fung *et al.* 1999; Chandrajith *et al.* 2007). The use of IC to determine water soluble fluoride is documented for water samples (Smee *et al.* 1978; Saha and Kundu 2003), but to the author's knowledge, fluoride determination in tea infusions is to a limited extent (Michalski 2006).

In analyses, a sample preparation protocol should be established. This will ensure all samples are treated the same and are representative of the investigated material (Keith 1991). Quality assurance measures must be undertaken to ensure the accuracy and precision, hence the validation of the data. This should include replicate analysis, control samples, blanks and analysis of certified reference materials (CRM's) with a similar matrix to the sample (Keith 1991). Measures should be taken to avoid cross contamination and samples must be prepared and stored in a uniform manner to keep constants the same throughout the experiment (Keith 1991). Glassware should be acid washed, using a solution of diluted nitric or hydrochloric acid, to remove any trace elements that could be bound to the glass surface (Kebbekus 2003).

The aim of this chapter is to develop methods to determine total fluoride in tea products, infusions, plants and soil. Towards this aim the following objectives were addressed:

- To develop a suitable/robust method for the determination of fluoride through suitable sample selection and instrumentation in a variety of tea samples and soil.
- To critically assess the validation of data using quality assurance for the analysis of total fluoride content.

## 2.2 Materials and Methods

A general order for the structure of Section 2.2 is summarised in Figure 2.3.

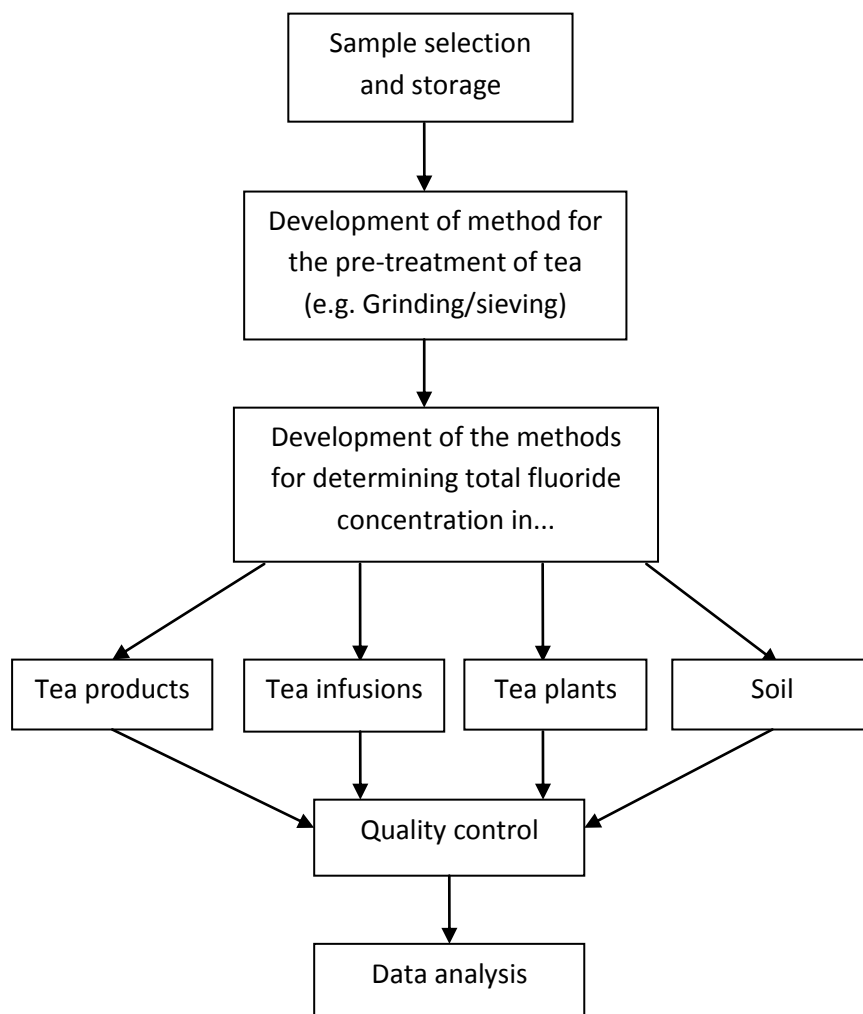


Figure 2.3 Flow chart of materials and methods section

## **2.2.1 Tea products (and infusions)**

### **2.2.1.1 Materials**

Three tea products were selected for the method development - Unilever PG Tips loose leaf tea and tea bags, and Clipper Ltd Organic Pure Green loose tea leaf, all UK brands (Nielson 2008). Tea leaves were removed from the tea bags, where necessary, and treated in the same way as the loose leaves. CRM's included tea GBW07605 (certified fluoride value  $322 \pm 31 \text{ mgkg}^{-1}$ ), NIST2695 timothy grass low level and high level (certified fluoride value  $64.8 \pm 8 \text{ mgkg}^{-1}$  and  $277 \pm 27 \text{ mgkg}^{-1}$ , respectively), from LGC Ltd.

### **2.2.1.2 Methods**

#### **a. Sample selection and storage**

PG tea leaf and PG tea bags were selected to develop a suitable pre-treatment prior to determining total fluoride concentration. PG tea leaf was used in the total fluoride method development; together with certified reference materials, tea GBW07605, low and high level timothy grass NIST2695. Clipper green leaf tea, PG tea leaf and PG tea bag products were selected for the initial method development for total fluoride in tea infusions.

All reagents used were of analytical grade or above and glassware was acid washed with 10% (w/v) nitric acid and triple rinsed with de-ionised water.

## **b. Pre- treatment of tea leaf product prior to analysis**

To produce a representative and uniform sample particle size, various methods of grinding and sieving were applied to PG Tips leaf and PG tea bags.

### **i. Cryogenic grinding**

Pestle and mortars were pre-cooled in a -80 °C freezer for a minimum of 2 hours prior to grinding. The tea leaf product was placed in the mortar and liquid nitrogen poured in. The frozen leaf material was ground to a fine powder using the pestle (Brewin *et al.* 2001). A separate pestle and mortar was used for each sample.

### **ii. Grind with mill (repetitive sample cleaning)**

Tea leaf samples were milled using a laboratory mill (IKA M20 Labortechnik, Germany). A sieve brush was used to clean as much residue from the mill. A small portion of the next sample was milled, and this was discarded. This step was repeated twice more and the third sample was collected for analysis.

### **iii. Grind with mill, acetone clean up**

After grinding with the laboratory mill (IKA M20 Labortechnik, Germany), clean up between samples comprised of brushing the mill free of residue with a sieve brush. This was followed by rinsing with deionised water, an acetone rinse and drying with tissue paper.

### **iv. Tea leaves particle size**

Different tea leaf particle sizes were tested to determine whether sieving had an effect on total fluoride concentration determination. After the samples were milled, a sub-sample was placed into a Kraft bag ready for analysis, referred to as (i) not sieved. The remainder of the milled product was put through a stainless steel sieve



(mesh size <125µm) and stored in Kraft bags as (ii) what was collected in the sieve pan, particle size <125µm and (iii) what was left in the sieve, particle size >125µm.

#### **v. Storage**

All samples were placed into Kraft bags (made of wet strength brown paper) and dried at 60 °C for 16 hours prior to analysis (Fung *et al.* 2003). Once dried to constant weight, samples were stored in glass desiccators with silica gel, which was regenerated on a weekly basis.

### **c. Development of method for total fluoride concentration in tea products**

#### **i. Microwave assisted digestions**

This method was developed using a Milestone Ethos Ez closed vessel microwave digester (Milestone 2011). The following methods were adapted and summarised in Table 2.1.

#### ***Acid and peroxide Method***

Huang *et al.* (2004) used nitric acid to digest various plant materials, including tobacco, apple, clover and eucalypt leaves, with an open-vessel microwave system for the analysis of calcium, potassium, magnesium, phosphorus, sulphur, boron, copper, manganese and zinc. Although fluoride was not analysed by Huang *et al.* (2004), the breakdown of tea as a plant material could still be achieved. The only modification to the method in this study was the use of closed vessels in the digestion, and a reduction of sample weight. Samples (0.2 g) were weighed accurately to 4 decimal places into the reaction vessel. Concentrated nitric acid, 69% (5 ml) was added and left for 16 hours to decrease the vigorous reaction under microwave heating (Huang *et al.* 2004). Digestion was carried out as described in Table 2.1. Once cooled the pH was adjusted to pH 5 with potassium hydroxide

solution and transferred, quantitatively, to 25 ml volumetric flasks with deionised water.

#### ***TMAH Method***

Watts *et al.* (2002) determined iodide with alkali digestion using a conventional oven method; therefore, this was adapted for the halogen, fluoride. The digestion time was reduced from the original method because a microwave digester was used rather than a conventional oven. The samples (0.25 g) were weighed accurately to 4 decimal places into the reaction vessel. Tetramethylammonium hydroxide (TMAH), (5 ml) was added to each sample and the digestion carried out as described in Table 2.1. Once cooled the sample was transferred quantitatively to 25 ml volumetric flasks with deionised water.

#### ***Nitric acid Method***

Al-Merey (2004) compared ultrasonication against Teflon bomb digestion for fluoride extraction in plant samples including ground chick pea grain and beech leaves. The Teflon bomb method was followed but adapting with microwave digestion instead of conventional oven heating. Samples (0.25 g) were weighed accurately to 4 decimal places into the reaction vessel. Concentrated nitric acid (0.5 ml) was added to each sample and the digestion carried out as described in Table 2.1. Once cooled the pH was adjusted to pH 5 with potassium hydroxide solution and transferred, quantitatively, to 25 ml volumetric flasks with deionised water.

The sample solutions for all three methods were filtered through 0.45 µm cellulose nitrate syringe filters and transferred into 100 ml polypropylene containers ready for analysis by ion chromatography.

Table 2.1 Adapted microwave digestion methods for the analysis of total fluoride concentration

Method	Sample weight	Reagent	Digestion temperatures and times	Reference
Acid and peroxide	0.2 g	5 ml conc. nitric acid 16 hours at room temperature	75 °C for 10 min; 109 °C for 15 min; cool for 15 min. Add 1 ml 30% hydrogen peroxide, heat at 109 °C for 15 min.	Huang <i>et al.</i> (2004)
TMAH	0.25 g	5 ml Tetramethylammonium hydroxide	80 °C for 20 min.	Watts <i>et al.</i> (2002)
Nitric acid	0.5 g	5 ml conc. nitric acid	120 °C for 20 min.	Al-Merey (2004 )

## ii. Alkali fused digestions

Alkali fusion is an alternative to conventional acid digestion and is favoured for use with geological samples (Jackson 2000). Samples are mixed with an alkaline flux to form a fusion and heated to ash in a furnace. When cooled the flux is dissolved in an appropriate solution for analysis (Jackson 2000).

Two methods for fluoride analysis, solid potassium hydroxide Method and sodium hydroxide Method (Metrohm 2008; Sparks *et al.* 1996) were compared using PG leaf tea and CRM tea GBW07605. Samples were milled and sieved to <125 µm and pre-dried at 60 °C for 16 hours prior to analysis (Fung *et al.* 2003). The additional aqueous potassium hydroxide Method was an adapted method based on the solid potassium hydroxide Method and sodium hydroxide Method, Table 2.2.

### ***Solid potassium hydroxide Method***

Metrohm (2008) is a standard method for the analysis of total fluoride in tea. Samples (0.25 g) were weighed accurately to 4 decimal places into a nickel crucible

and 0.5 g of potassium hydroxide powder added to each. Fusion was carried out using the oven and furnace times stated in Table 2.2. Once the sample was ashed and cooled, the contents were dissolved and transferred quantitatively into 50 ml volumetric flasks with de-ionised water.

#### ***Sodium hydroxide Method***

Sparks *et al.* (1996) describes a method for soil analysis, but it has been used by authors for determining fluoride in vegetation (McQuaker and Gurney 1977; Fung *et al.* 1999). Samples (0.5 g) were accurately weighed to 4 decimal places into a 50 ml nickel crucible and 6 ml of 17 M sodium hydroxide was added. Crucibles were placed in an oven at 100 °C for 60 minutes and transferred into a furnace using the conditions stated in Table 2.2. Once cooled the ash content was dissolved in 10 ml de-ionised water and warmed on a hotplate. The samples were adjusted to pH 8 to 9 with approximately 8 ml of concentrated hydrochloric acid (37%). Dissolved samples were transferred quantitatively to 100 ml volumetric flasks with de-ionised water.

#### ***Aqueous potassium hydroxide Method***

A total of 0.5 g tea sample was weighed accurately to 4 decimal places into a nickel crucible and 2 ml of 50% (w/v) potassium hydroxide solution added. Crucibles were agitated to ensure the tea was uniformly mixed (Sparks *et al.* 1996). The crucible was placed on a hotplate calibrated to 100 °C for 30 minutes until fusion occurred and transferred to a muffle furnace set at 300 °C. Temperature was incremented at a rate of 50 °C every 15 minutes until reaching 600 °C where it was held for a further 30 minutes (Metrohm 2008). Once removed and cooled, the residue was transferred quantitatively to a 50 ml volumetric flask with deionised water.

Table 2.2 Alkali fused digestion methods for the analysis of total fluoride concentration in tea products

Method	Sample weight	Alkali	Oven and furnace temperatures and times	Reference
Solid potassium hydroxide	0.25 g	0.5 g potassium hydroxide	Heat in furnace at 200 °C for 15 min; raising by 50 °C every 15 min until reaching 500 °C and holding for 10 min.	Metrohm (2008)
Sodium hydroxide	0.5 g	17M sodium hydroxide	Oven at 100 °C for 60 min; furnace at 300 °C, raising by 50 °C every 15 min until reaching 600 °C and holding for 30 min.	Sparks <i>et al.</i> (1996)
Aqueous potassium hydroxide	0.5 g	2ml 50% (w/v) potassium hydroxide solution	Dry on hotplate at 100 °C for 30 min; heat in furnace at 300 °C for 15 min; raising by 50 °C every 15 min until reaching 600 °C and holding for 30 min.	Adaptation of Sparks <i>et al.</i> (1996) and Metrohm (2008)

Samples from each of the methods were filtered through 0.45 µm cellulose nitrate syringe filters and transferred 30 ml polystyrene sterile universal containers ready for analysis by ion chromatography.

#### **d. Development of method for total fluoride concentration in tea infusions**

##### **i. Infusion preparations**

##### ***Trial infusion preparation for IC***

After drying at 60 °C for a minimum of 16 hours to a constant weight (Fung *et al.* 2003), 2 g of un-milled PG tea leaf was accurately weighed to 4 decimal places into a 250ml Erlenmeyer flask Boiling deionised water (100 ml) was added and the flask capped with foil. This was repeated in triplicate. Each flask was infused at either 2, 10 and 30 minutes, to represent typical brewing times, and kept between 85 and 90 °C

in a water-bath (Duckworth and Duckworth 1978; Fung *et al.* 1999; Shu *et al.* 2003). The infusion was filtered through a 0.45 µm cellulose nitrate syringe filter into a 30 ml polystyrene sterile universal container for immediate analysis by IC.

#### ***Trial infusion preparation for ISE***

A dried un-milled Clipper green tea sample (1 g) was accurately weighed to 4 decimal places, into a 250 ml Erlenmeyer flask. Boiling deionised water (100 ml) was added and the flask was swirled once and capped with foil to prevent evaporation. The flask was kept between 85 and 90 °C in a water-bath for 2 minutes (Duckworth and Duckworth 1978; Fung *et al.* 1999; Shu *et al.* 2003). The suspension was filtered through a Whatman No.1 filter paper (Fung *et al.* 1999). The filtrate was stored in a 30 ml polystyrene sterile universal container ready for immediate analysis.

#### ***Tea leaf (un-milled) preparation***

Repeat ‘coning and quartering’ was used to produce a representative test sample (McNaught and Wilkinson 1997), followed by drying at 60 °C for a minimum of 16 to a constant weight (Cao *et al.* 2003). Samples (2 g) were accurately weighed to 4 decimal places, into 250 ml Erlenmeyer flasks. 100ml boiling deionised water was added and each flask swirled once and capped with foil. The flasks were kept between 85 - 90 °C in a water-bath and infused for either 2, 10 or 30 minutes (Duckworth and Duckworth 1978; Fung *et al.* 1999; Shu *et al.* 2003). After each timed interval, suspensions were filtered through Whatman No.1 filter papers (Fung *et al.* 1999). The filtrates were stored in 30 ml polystyrene sterile universal container ready for immediate analysis.

#### ***Tea leaf (milled and sieved to <125 µm) preparation***

Samples were milled and sieved to <125 µm and pre-dried at 60 °C for 16 hours prior to analysis (Fung *et al.* 2003). Tea samples (0.5 g) were accurately weighed to 4 decimal places into 100 ml Erlenmeyer flasks. Boiling deionised water (25 ml) was

added and the flasks swirled once and capped with foil. The flasks were kept between 85 - 90 °C in a water-bath and infused at 2, 10 and 30 minutes respectively, followed by filtering the suspension through a Whatman No.1 filter paper (Fung *et al.* 1999). The filtrates were stored in 30 ml polystyrene sterile universal container ready for immediate analysis.

## **e. Instrumentation for fluoride detection**

### **i. Pre-treatment samples by IC**

Samples were digested using alkali fused digestion, aqueous potassium hydroxide method, described in Table 2.2 (Metrohm 2008). Although the ion selective electrode (ISE) method is favoured in literature because it is more economical and requires less time in setting up and analysing samples (Radiometer 2012), ion chromatography (IC) was chosen as the method of detection because this was used in the Metrohm (2008) methodology. A Metrohm IC basic 792 with conductivity detector was used. Data was collected using Metrohm software 792 PC Software 792. Chromatographic column Metrohm A Supp 5-250 column with Metrohm 4/5 guard was selected using eluent, 3.2 mmol/l sodium carbonate and 1.0mmol/l sodium hydrogen carbonate, flowing at 0.7 ml/min. Total run time was 32 minutes and the injection volume was 20 µl.

The IC was calibrated using 1, 2, 3, 4 and 5 mg l<sup>-1</sup> fluoride solutions prepared from 1000 mg l<sup>-1</sup> fluoride standard stock solution (Fisher Scientific) in deionised water. The IC was calibrated every 12 samples, to produce a calibration curve with coefficient of determination (R<sup>2</sup>) value. A chromatogram for each sample provided a value of fluoride in mg l<sup>-1</sup>. The following calculation converted fluoride to mg kg<sup>-1</sup>.

$$[\text{F}^-] \text{ mg kg}^{-1} = \frac{[\text{F}^-] \text{ mg l}^{-1} \times \text{dilution factor (ml)}}{\text{Weight of sample (g)}}$$

## **ii. Tea leaf products, tea plants and soils by IC**

A Metrohm IC basic 792 with conductivity detector was used following instrumental conditions and calibration described in Section 2.2.1.2 e (i).

## **iii. Tea infusions**

### ***IC***

Ion chromatography was initially tested as this was the method used for the analysis of total fluoride in the product (Michalski 2006). Michalski (2006) reported successful separation of fluoride from tea infusions using ion chromatograph. All samples were filtered through 0.45µm cellulose nitrate syringe filter prior to injection. Instrument conditions and calibration were as described Section e (i).

### ***ISE***

A total ionic strength adjustment buffer (TISAB) was prepared using 58 g sodium chloride, 12 g sodium citrate, 57 ml acetic acid, adjusted to pH 5.0 - 5.5 with sodium hydroxide and made up to 1000 ml volume with deionised water (Sparks *et al.* 1996). The ISE was calibrated using standards of 0.1, 1.0, 10.0, 100 mg l<sup>-1</sup> fluoride (w/v), prepared from a sodium fluoride stock solution of 1000 mg l<sup>-1</sup>. Buffer was added to all samples and standards at a concentration of 50% prior to fluoride analysis to eliminate interference from ions such as OH<sup>-</sup>, Fe<sup>2+</sup> and Mn<sup>2+</sup> (Rundle 2013). Readings were taken for both standards and samples after a stirring period of 1 minute to ensure thorough mixing with the buffer.

The calibration was plotted on a logarithmic scale and the sample results anti-logged, taking the weight and dilution into consideration to give the fluoride concentration in mg l<sup>-1</sup> using Excel™.



## **2.2.2 Tea Plants**

### **2.2.2.1 Materials**

Tea CRM tea GBW10016 (certified fluoride value  $57 \pm 15 \text{ mgkg}^{-1}$ ) and NIST2695 timothy grass high level (certified fluoride value  $277 \pm 27 \text{ mgkg}^{-1}$ ), were used, all purchased from LGC Ltd.

### **2.2.2.2 Methods**

#### **a. Development of a method for the analysis of total fluoride in tea plants**

As the CRM NIST2695 Timothy grass high level and low level were vegetative plant materials and have previously been analysed using the method described in Section 2.2.1.1, the new tea CRM GBW10016 was determined as five replicates together with eight replicate analyses of Timothy grass high level NIST2695.

#### **i. Tea plant preparation**

The method for total fluoride in tea products, Section 2.2.1.2 c (ii), Table 2.2, was followed using alkaline fusion with aqueous potassium hydroxide, as an adaptation of Sparks *et al.* (1996) and Metrohm (2008).

#### **ii. Instrumentation**

Instrumentation and settings followed the total fluoride in tea products, Section 2.2.1.2 e (i) (Metrohm 2008).

### **2.2.3 Soils**

#### **2.2.3.1 Materials**

Soil CRM NCS ZC73006 (certified fluoride value  $652 \pm 48 \text{ mgkg}^{-1}$ ) was used, purchased from LGC Ltd.

#### **2.2.3.2 Methods**

##### **a. Development of method for the analysis of total fluoride in soils**

The soil CRM NCS ZC73006 was analysed as eight replicates to determine the percentage recovery of fluoride and accuracy.

##### **i. Soil preparation**

As for the tea plants, Section 2.2.2, the methodology adopted was alkaline fusion with aqueous potassium hydroxide, as an adaptation of Sparks *et al.* (1996) and Metrohm (2008), Section 2.2.1.2 c (ii), Table 2.2.

##### **ii. Instrumentation**

Instrumentation and settings followed the total fluoride in tea products by IC, Section 2.2.1.2 e (i) (Metrohm 2008).

##### **b. Development of a method for water soluble fluoride in soils**

##### **i. Soil preparation**

Ten replicates of soil CRM NCS ZC73006 were extracted based on the method described by Saikat (2004) where 0.5 g of <2 mm air dried soil were accurately

weighed in to 50 ml screw capped glass bottles and 25 ml of de-ionised water was added. The bottles were shaken for an hour at 70 rpm. The mixture was filtered through 0.45 µm cellulose nitrate syringe filters.

## **ii. Instrumentation**

Instrumentation and settings followed the total fluoride in tea products by IC, Section 2.2.1.2 e (i) (Metrohm 2008).

### **2.2.4 Quality control**

Instrumental limit of detection (LOD) and limit of blank (LOB) for the IC and ISE were determined by analysing ten 0.02 mg l<sup>-1</sup> fluoride standard solutions, followed by ten blank de-ionised water samples (Armbruster and Pry 2008).

#### **2.2.4.1 Ion chromatography**

For pre-treatment analysis, all samples were determined in triplicate. For the development of the method for total fluoride in tea products, triplicate extractions were carried out. A sample blank and tea CRM GBW07605 were included with every batch of 10 samples.

For the aqueous potassium hydroxide adapted method, the inclusion of certified reference material high and low level timothy grass (NIST 2695) were included to validate the out of date tea CRM. Precision of the IC was tested by multiple injections of the same sample and by running all the samples in triplicate.

For the tea plant and soil methods, up to ten replicate analysis of a CRM with a similar matrix were used.

#### **2.2.4.2 Ion selective electrode**

A CRM for tea infusions was not available, instead certified reference material NCSZC76304 (fluorine in water) was used. A prepared standard of 3 mg l<sup>-1</sup> fluoride was analysed with every 10 samples, together with a sample blank of deionised water. Clipper green tea, in the initial infusion development was determined in quadruplicate and all the other samples were determined in triplicate.

#### **2.2.5 Data analysis**

Data analysis was carried out using software packages Microsoft Excel 2007 and IBM SPSS 19. Descriptive statistics were used to summarise numerical and graphical means, standard deviations and coefficient variations of the measured continuous data, for fluoride concentrations, using Microsoft Excel. To determine whether data was normally distributed, the Shapiro-Wilk test was carried out using SPSS, which was appropriate for small sample sizes (<50 samples) (Pallant 2013). If the data proved to be significant ( $p > 0.05$ ), a parametric normal distribution was assumed (Pallant 2013). Therefore, Students t-test (Excel) and ANOVA with Bonferroni multiple comparison test (SPSS) were suitable to determine any significant results at  $p < 0.05$ . Confidence levels describing significant differences of results were reported to be either significant at 0.05 (95%), very significant at 0.01 (99%) or highly significant at 0.001 (99.9%) (Salkind 2008). R-squared, coefficient of determination ( $R^2$ ) was used to measure the closeness of data to a fitted regression line (Excel). Where  $R^2$  is any value between 0 - 1, and a value of 1 indicates a perfect fit of the data with the regression line (Carlberg 2011).

## 2.3 Results

### 2.3.1 Tea products (and infusions)

#### a. Pre- treatment of tea products

Total fluoride concentration ( $\text{mgkg}^{-1}$ ) for PG leaf tea and PG tea bags using the different preparation methods, determined by IC are shown Table 2.3. There were significant differences for fluoride concentration between all the grind methods and particle sizes for PG leaf tea and PG tea bags ( $p>0.05$ ) with the exception of particle size  $<125\mu\text{m}$ , which was not significantly different to  $>125\mu\text{m}$  and 'not sieved' PG leaf tea samples.

Table 2.3 Total fluoride in PG leaf tea and tea bags by comparing grind methods and particle size (mean  $\pm$  SD,  $n=3$ )

Grind method	Sieve size	PG leaf tea [F-] $\text{mgkg}^{-1}$	PG tea bag [F-] $\text{mgkg}^{-1}$
Cryogenic grinding	Not sieved	$307 \pm 7$	$362 \pm 11$
Mill, repetitive sample clean up	Not sieved	$312 \pm 5$	$370 \pm 7$
Mill, acetone clean up	Not sieved	$306 \pm 13$	$370 \pm 10$
Cryogenic grinding	$<125\mu\text{m}$	$320 \pm 8$	$372 \pm 13$
Mill, repetitive sample clean up	$<125\mu\text{m}$	$321 \pm 4$	$384 \pm 6$
Mill, acetone clean up	$<125\mu\text{m}$	$331 \pm 4$	$386 \pm 16$
Cryogenic grinding	$>125\mu\text{m}$	$318 \pm 10$	$354 \pm 29$
Mill, repetitive sample clean up	$>125\mu\text{m}$	$315 \pm 8$	$367 \pm 12$
Mill, acetone clean up	$>125\mu\text{m}$	$307 \pm 8$	$363 \pm 13$

### **2.3.2 Total fluoride determination in tea products, plants and soils**

#### **a. Microwave assisted digestions**

Appropriate digestions of the samples were not achieved for the nitric and perchloric, TMAH and nitric acid Methods.

#### **b. Alkali fused digestions**

##### ***Solid potassium hydroxide Method***

Fluoride concentration recoveries (%) for the two independent digestions of the tea CRM were 77.8% and 124%, reflecting poor accuracy using the solid potassium hydroxide Method, Table 2.4 (Metrohm 2008).

##### ***Sodium hydroxide Method***

Fluoride could not be quantified as a successful chromatographic separation from the alkali digestion of the tea CRM was not achieved due to interfering peaks.

##### ***Aqueous potassium hydroxide Method***

Fluoride concentrations for the CRM's using this method are given in Table 2.4. High level timothy grass CRM gave the lowest recovery of 99%, with low level timothy grass CRM and tea CRM showing 100% recoveries. Validation of the 'out of date' tea CRM, was achieved by comparing the recovery percentage with the new timothy grass CRM's, which gave accurate results.

Table 2.4 % Recovery of fluoride concentration for solid vs. aqueous potassium hydroxide methods (mean  $\pm$  SD, n=3)

Solid potassium hydroxide Method (Metrohm 2008)			
Sample	Certified concentration mgkg <sup>-1</sup>	Measured fluoride mgkg <sup>-1</sup>	% recovery
Tea CRM (GBW07605)	320 $\pm$ 31	249	77.8
Tea CRM (GBW07605)	320 $\pm$ 31	399	124
Aqueous potassium hydroxide Method (adapted from Metrohm 2008)			
Tea CRM (GBW07605)	320 $\pm$ 31	320	100
Low level timothy grass CRM	64 $\pm$ 8	64	100
High level timothy grass CRM	277 $\pm$ 27	274	99

The aqueous potassium hydroxide Method was validated further by determining the total fluoride concentration in PG tea leaf, PG tea bags and Clipper green tea leaf, analysing the tea CRM (GBW07605) in the sample batch, Table 2.5. All of the standard deviations for these determinations were less than 2.5% from the measured value.

Table 2.5 Total fluoride concentration in samples using the aqueous potassium hydroxide Method (mean  $\pm$  SD, n=3)

Sample	Measured [F <sup>-</sup> ] mgkg <sup>-1</sup>
PG leaf tea	325 $\pm$ 5
PG tea bags	382 $\pm$ 6
Clipper green leaf tea	393 $\pm$ 6
Tea CRM (GBW07605)	322 $\pm$ 8 (101% recovery)

To determine the precision of the IC instrumentation using conditions set out in Section 2.2.1.2 e, chromatographic peak area was compared using multiple injections of the same sample. Data is given in Table 2.6. Peak area ranged from 98.37 to 103.68, with the mean and standard deviation calculated as  $100.74 \pm 2.17$ . The peak area was consistent with no significant differences ( $p>0.05$ ).

Table 2.6 Peak areas from replicate injections using IC

Injection	Peak area
1	102.54
2	103.68
3	102.74
4	99.4
5	98.37
6	98.89
7	99.56
Mean and SD (n=7)	$100.74 \pm 2.17$

The calibration curve was produced using the IC software and gave a coefficient of determination ( $R^2$ ), linearity of the curve not less than 0.9997 (Figure 2.4), indicating an accuracy of <1% (O'Haver 2011).

The limit of blank and limit of detection were calculated as 0.13 and 0.14  $\text{mg l}^{-1}$ , respectively (Armbruster and Pry 2008).



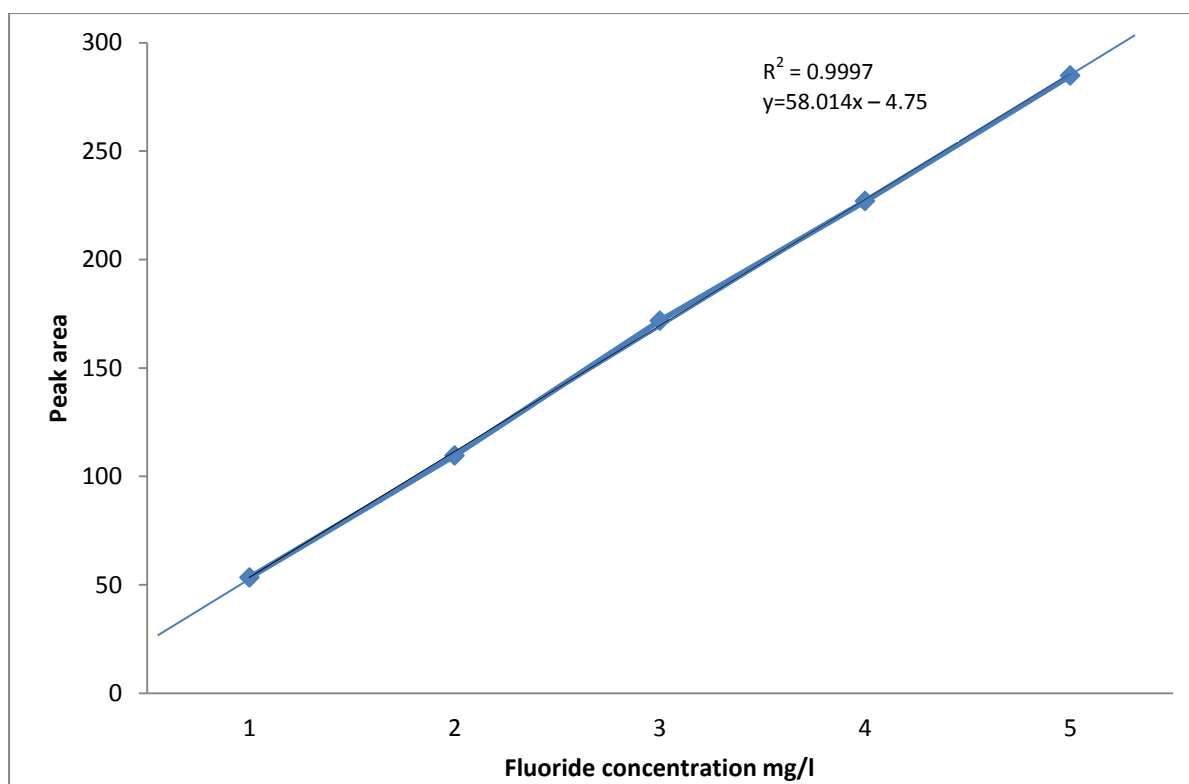


Figure 2.4 Calibration curve for fluoride concentration using IC

### **2.3.3 Total fluoride analysis in tea infusions**

#### **a. Infusions by IC**

Results for fluoride concentration in tea infusions were not achieved using IC. The chromatographic separation of fluoride from other components was unsuccessful using IC instrumentation.

#### **b. Infusions by ISE**

##### **i. Trial infusion**

Fluoride concentration ranged from 2.5 to 3.5 mg l<sup>-1</sup> with a mean and standard deviation of 3.0 ± 0.4 mg l<sup>-1</sup>. A CRM was not included with this batch as this was to determine whether results were in range with published literature. Although the ISE

was calibrated, quality control was not incorporated at this stage as this was developed further to include validation in Section 2.3.3 b ii.

## ii. Tea leaf, un-milled

PG leaf tea, PG tea bags and Clipper green leaf tea were analysed for fluoride concentration, using water CRM and a 3 mg l<sup>-1</sup> or 5 mg l<sup>-1</sup> fluoride standard checks. Results are given in Table 2.7. Fluoride becomes significantly ( $p < 0.05$ ) more soluble with increased infusion time for both PG leaf tea and PG leaf bags.

Percentage recovery of fluoride concentration for the water CRM and fluoride standard checks are given in Table 2.7. The calibration curves gave the coefficient of determination ( $R^2$ ) of 0.9995 and 0.9993, respectively, for PG leaf tea and PG leaf bags analyses. This indicates the linear regression successfully fits the data with an accuracy of <1% (O'Haver 2011).

Table 2.7 Total fluoride concentration in un-milled tea infusions (mean  $\pm$  SD, n = 3)

Infusion time (min)	PG leaf tea* [F <sup>-</sup> ] in infusion mg l <sup>-1</sup>	PG tea bags* [F <sup>-</sup> ] in infusion mg l <sup>-1</sup>	Clipper leaf tea* [F <sup>-</sup> ] in infusion mg l <sup>-1</sup>
2	3.9 $\pm$ 0.3	5.0 $\pm$ 0.4	4.3 $\pm$ 0.7
10	5.0 $\pm$ 0.2	5.2 $\pm$ 0.2	6.2 $\pm$ 0.7
30	5.3 $\pm$ 0.2	6.4 $\pm$ 0.3	6.4 $\pm$ 0.4
Water CRM (1 mg l <sup>-1</sup> )	0.97 mg l <sup>-1</sup> (97% recovery)	1.04 mg l <sup>-1</sup> (104% recovery)	0.97 mg l <sup>-1</sup> (97% recovery)
3 mg l <sup>-1</sup> or 5 mg l <sup>-1</sup> fluoride solution	5.72 mg l <sup>-1</sup> (114% recovery)	4.96 mg l <sup>-1</sup> (99% recovery)	2.83 mg l <sup>-1</sup> (94% recovery)

### iii. Milled tea leaf (<125µm)

Particle size of <125µm and fluoride solubility in the infusions of PG leaf tea, PG tea bags and Clipper green leaf tea results are given in Table 2.8. Fluoride concentration ranged from 4.6 to 5.4 mg l<sup>-1</sup> for PG leaf tea, 4.7 to 6.0 mg l<sup>-1</sup> in PG tea bags and 4.3 to 7.1 mg l<sup>-1</sup>. There was no significant difference ( $p>0.05$ ) between fluoride concentration and infusion time for any of the samples.

A standard check using a prepared 3 mg l<sup>-1</sup> fluoride solution gave a 93 and 94% recovery and water CRM (NCSZC76304) gave 97 and 104% fluoride recovery in the batches of analyses. The coefficient of determination ( $R^2$ ) for the calibrations were 0.9991 and 0.999, respectively, for PG leaf tea and PG tea bags analyses, indicating a successful linear regression of data with an accuracy error of <1% (O'Haver 2011).

Table 2.8 Total fluoride concentration in tea infusions prepared with <125 µm sample particle size (mean ± SD, n=3)

Infusion time (min)	PG leaf tea* [F-] in infusion mg l <sup>-1</sup>	PG tea bags* [F-] in infusion mg l <sup>-1</sup>	Clipper leaf tea* [F-] in infusion mg l <sup>-1</sup>
2	5.2 ± 0.1	5.8 ± 0.1	4.6 ± 0.3
10	5.1 ± 0.5	5.8 ± 0.1	4.8 ± 0.1
30	5.0 ± 0.2	5.0 ± 0.4	5.0 ± 0.1
Water CRM (1 mg l <sup>-1</sup> )	0.97 mg l <sup>-1</sup> (97% recovery)	1.04 mg l <sup>-1</sup> (104% recovery)	0.97 mg l <sup>-1</sup> (97% recovery)
3 mg l <sup>-1</sup> fluoride solution	2.80 mg l <sup>-1</sup> (93% recovery)	2.80 mg l <sup>-1</sup> (93% recovery)	2.83 mg l <sup>-1</sup> (94% recovery)

Comparing all infusion times together as a set for the 2, 10 and 30 minutes, a significant difference ( $p < 0.05$ ) in fluoride concentration was observed between the un-milled and  $< 125 \mu\text{m}$  particle sizes for both PG leaf tea and PG tea bags.

The limit of detection for the ISE was calculated as  $0.03 \text{ mg l}^{-1}$ , and limit of blank was  $0.02 \text{ mg l}^{-1}$ , following methods described by Armbruster and Pry (2008).

#### **2.3.4 Total fluoride in tea plants**

Replicate analysis of CRM NIST2695 Timothy grass high level gave a mean fluoride concentration of  $261 \pm 11 \text{ mg kg}^{-1}$  ( $n=8$ ) which was within the certified value range of  $277 \pm 27 \text{ mg kg}^{-1}$ , with a percentage recovery of 94.2%. For the new tea CRM GBW10016, the mean concentration was  $70 \pm 7 \text{ mg kg}^{-1}$ , within the certified range of  $57 \pm 115 \text{ mg kg}^{-1}$ .

#### **2.3.5 Total and water soluble fluoride in soils**

Analysis of the soil CRM NCS ZC73006 gave a mean fluoride concentration of  $639 \pm 15 \text{ mg kg}^{-1}$  ( $n = 8$ ), which agreed with the certified value of  $652 \pm 48 \text{ mg kg}^{-1}$  and this gave a percentage recovery of 98.0%.

For water soluble fluoride CRM NCS ZC73006, the mean concentration was  $26.6 \pm 2.4 \text{ mg kg}^{-1}$  ( $n=10$ ), which indicated good precision of the method. There is not a certified value for water soluble fluoride, so this CRM value was used when analysing further soils.

### **2.3.6 Quality control**

All results for the CRM recoveries, standard recoveries, blanks and replicates for all methods are included in the specific Sections 2.3.1 to 2.3.5 above.

## **2.4 Discussion**

### **2.4.1 Pre- treatment of tea product**

The sample pre-treatment for choice of grinding method were significantly different ( $p < 0.05$ ), so the selection was based on the results with the lowest standard deviation. Grinding and cleaning the mill by repetitive sample flushing, together with particle size of  $< 125\mu\text{m}$  was chosen for the analysis of total fluoride in tea products and plant material. This was based on being the most precise method by having the lowest standard deviation. Sample cross contamination needed to be prevented and this was achieved by cleaning the mill by repetitive sample flushing, reflecting good precision in the results (Keith 1996).

### **2.4.2 Total fluoride determination in tea products, leaf and soil**

#### **a. Microwave assisted digestions**

Digesting the tea products using the nitric and perchloric acid, TMAH and nitric acid Methods, Table 2.1 proved unsuccessful for the determination of total fluoride concentration. The choice of reagents in all three Methods caused chromatographic separation problems with IC. Interfering anions such as chloride in hydrochloric acid in Method A and nitrate from nitric acid in Method B, affected the peak separation. Overloading of the column was observed; therefore, fluoride could not be detected. Using acids also made it difficult to optimise the pH to above pH 3, suitable for the

chromatographic column (Metrosep 2008). To achieve this, the samples required neutralisation where an amount of alkali was added, but this gave variation in the sample final volumes.

The organic nature of the TMAH reagent used for digestion in the TMAH Method was unsuitable for IC. Instead, ICP-AES was the favoured method of detection, as used by Watts *et al.* (2002) for iodide; however, this instrumentation was not available. Organic interferences can be overcome using multi-dimensional matrix-elimination ion chromatography, which involves attaching a series of columns together (Zakaria *et al.* 2011). Equipment limitations and the costs involved in reproducing this set up prevented this from being attempted.

Generally, acid digestion is more suitable for extracting cations, such as cobalt, copper, lead and cadmium using IC in environmental and biological samples (Jackson 2000; Shaw and Haddad 2004). Stevens *et al.* (1995) and Malde *et al.* (1997) compared acid digestion with alkali digestion for methods to analyse fluoride concentration in vegetation and oyster. Both groups of authors reported inconsistencies with the acid digestion, favouring alkali digestion, with Malde *et al.* (1997) reporting a 100 fold difference in fluoride from the oyster reference material (NIST1566a) between the two digestions. Although the analyses were carried out at different laboratories, Malde *et al.* (1997) stated the need for a standardised method to determine fluoride concentration in food. IC was not used for the instrumentation with any of the digestions in these studies, but ISE was adopted (Stevens *et al.* 1995; Malde *et al.* 1997).

## **b. Alkali fused digestions**

Alkali fused digestion for the analysis of total fluoride concentration in tea products was introduced using solid potassium hydroxide, sodium hydroxide and aqueous

potassium hydroxide Methods, Table 2.2. Precision and accuracy were not obtained using the solid potassium hydroxide Method (Metrohm 2008). The fusion of sample and alkali was incomplete in areas. Non-fused tea was observed to have ashed separately from the fused tea. The resulting inhomogeneity in the samples was thought to be the reason for the poor percentage recovery of fluoride in the tea CRM.

Sparks *et al.* (1996) used liquid alkali and this was reproduced in the sodium hydroxide Method. The pH of the sample required neutralising with hydrochloric acid for the requirements of the IC column. This resulted in excess chloride ions from the hydrochloric acid overloading the chromatographic column (Sparks *et al.* 1996). Only one sample was attempted to prevent column damage as they all contained concentrations of chloride. Fusion of these samples was observed to be homogenous as the liquid alkali improved mixing with the tea product. Adapting the solid potassium hydroxide Method and the sodium hydroxide Method, resulted in producing the aqueous potassium hydroxide Method by using 2 ml of 50% (w/v) potassium hydroxide solution, rather than 0.5 g of the solid form, to aid fusion.

Alkali fused digestion for the aqueous potassium hydroxide Method was successful in achieving good fluoride recoveries ranging from 95 to 100%. Adjusting the alkali to a liquid form and physically tapping the crucible ensured homogenous mixing with the tea sample. Malde (1997) agreed that the alkali digestion method was the most accurate, achieving a 104% fluoride concentration recovery when adopting the method for certified reference material oyster tissue (NIST1566a). Malde (1997) does not describe whether the alkali was in liquid or solid form. The fluoride concentration results for the black tea samples PG leaf tea and PG tea bags were 325 mgkg<sup>-1</sup> and 382 mgkg<sup>-1</sup>, respectively, and comparable to data by Fung *et al.* (1999) and Yi and Cao (2008) of 204 to 423 mgkg<sup>-1</sup> and 30 to 385 mgkg<sup>-1</sup>, respectively. Mean total fluoride concentration of Clipper green tea leaf was determined as 393 mgkg<sup>-1</sup>,

although higher than those published by Shu *et al.* (2004), this agreed with Fung *et al.* (1999) and was in range with those summarised by Yi and Cao (2008). Based on the reproducibility, precision and accuracy of the adapted method, incorporating IC detection, this technique was deemed successful for use in future analyses within this study.

### **2.4.3 Total fluoride analysis in tea infusions**

#### **a. Infusions by IC**

The preferred instrumentation was IC for the detection of fluoride in the infusions as this was to be adopted for the tea products. Michalski (2006) successfully separated fluoride from other anions in a tea infusion using IC, but although instrumentation and conditions matched the study, it failed to be reproducible. Possible organic acids present in the tea interfered with the anionic profile, therefore separation of the fluoride peak was not achieved (Peldszus 2006; Englehardt 2010).

#### **b. Infusions by ISE**

##### **i. Trial infusion**

Substantial studies describe using ISE for fluoride detection, especially for tea infusions, also known as water soluble fluoride (Duckworth and Duckworth 1978; Effendi and Wibowo 1984; Smid and Kruger 1985; Schamschula *et al.* 1988; Chan and Koh 1996; Gulati *et al.* 2003; Cao *et al.* 2004; Chandrajith *et al.* 2007; Sofuoglu and Kavcar 2008; Malinowska *et al.* 2008). Results were slightly higher for the Clipper green tea leaf (2.5 to 3.5 mg l<sup>-1</sup>) compared to green tea analysed by Wong *et al.* (2003), reporting a range of 1.2 to 1.7 mg l<sup>-1</sup> and Reto *et al.* (2008) who reported a range of 0.8 to 2.0 mg l<sup>-1</sup>. The results were in a similar range to literature and this formed the foundations of the method to be developed further, Section 2.3.3 b ii.



## **ii. Tea leaf, un-milled and milled (<125µm)**

Total fluoride concentration for all the un-milled tea infusions ranged from 3.5 to 5.5 mg l<sup>-1</sup> at 2 minutes, 4.9 to 7.1 mg l<sup>-1</sup> at 10 minutes and 5.1 to 6.9 mg l<sup>-1</sup> after 30 minutes, Table 2.7. This agrees with Fung *et al.* (1999) reporting 0.54 to 7.05 mg l<sup>-1</sup> and Yi and Cao (2008) who summarise a wider range of 0.0 to 33.4 mg l<sup>-1</sup>. In both of these studies, a greater number and range of tea types were analysed (Fung *et al.* 1999; Yi and Cao 2008). Malinowska (2008) reported similar fluoride leaching in tea infusions with varying brewing times which agrees with most of the findings in this study. Although infusing at 10 and 30 minutes would not represent typical consumer habit of tea preparation, these times were included to represent the extreme. However, use of a teapot may result in longer infusion periods and over steeping of teas compared to a 'one cup' preparation (Teablog 2011).

When the tea was milled to <125 µm, the solubility of the fluoride after 2 minutes was not significantly different ( $p>0.05$ ) to the concentration of fluoride in the 30 minute infusion. Therefore, infusing at 10 minutes at this particle size was chosen not to be assessed in future studies. Prior to milling, it was observed that different brands and tea types do not have a uniform particle size. For example, the particle size was visually observed to be finer in PG tea bags than the coarser PG tea leaf, Figure 2.5. Milling to <125 µm will provide data on differences in fluoride concentration between different tea types, whilst removing the particle size variable.

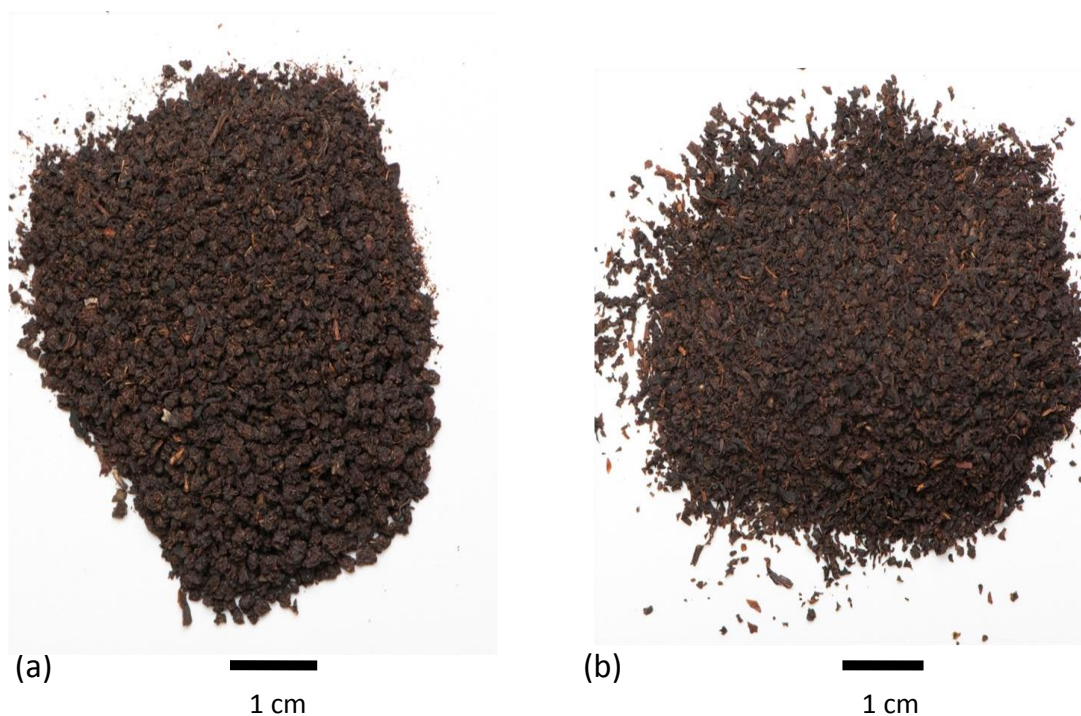


Figure 2.5 Visual comparison of different particle sizes between (a) PG leaf tea (b) and PG tea bags

#### **2.4.4 Total fluoride in tea plants and in soils**

Following the success of the aqueous potassium hydroxide method for tea products, this method was adopted for the tea plant materials and soils. The percentage fluoride recoveries of the CRM Timothy grass high level (NIST2695) and soil CRM (NCS ZC73006) of 94.2% and 98.0%, respectively, ensured that this method was suitable for plant materials and soils.

#### **2.4.5 Quality control**

The percentage fluoride recoveries for the various CRM's ranged from 95 to 100% using the aqueous potassium hydroxide Method with IC, indicating a high level of accuracy. For the ISE methodologies, CRM recoveries were over a wider range, from

93 to 114%, but also indicated accuracy. The methods were further validated using quality assurance techniques such as the inclusion of blanks and sample replicates. The successful digestion or extraction of fluoride from the tea product, infusion, plant material and soil, together with a multi-point calibration of the instrumentation used for the detection, ensured the accuracy and precision required for the methods to be accepted to determine fluoride concentration for the rest of this study (Riley and Rosanske 1996).

## 2.5 Chapter Summary

- Sample pre-treatment of the tea products and plant materials using a laboratory mill and cleaning using repetitive sample flushing avoided cross contamination and produced the lowest standard deviation between replicate sample analyses.
- Modifications were made to develop a novel approach based on Sparks *et al.* (1996) and Metrohm (2008) to digest tea products, soils and plants ready for elemental analysis. This involved changing the form of alkali to 50% (w/v) aqueous potassium hydroxide, which produced a homogenous sample fusion with CRM recoveries from 95 to 100%.
- A good chromatographic separation of fluoride from other anions was achieved using IC for total fluoride concentrations in tea products, soils and plants. ISE was a suitable detection method for fluoride concentration in tea infusions rather than IC. Both techniques produced accurate and precise results.
- For the un-milled tea infusions, total fluoride was analysed at 2, 10 and 30 minutes; reflecting typical and extreme brewing times (Teablog 2010). There were some significant increases in fluoride release with the different infusion times.

- There were no significant differences ( $p>0.05$ ) in fluoride concentration for PG leaf tea and PG tea bags between the infusion times of 2, 10 and 30 minutes when milled and sieved to  $<125\ \mu\text{m}$ . Therefore, for further samples in this study, the 10 minute infusion time was unnecessary.
- Percentage fluoride recovery ranged from 93 to 114% using prepared fluoride standards. Calibration curves gave the coefficients of determination ( $R^2$ ) of 0.9995 and 0.9993, suggesting an accuracy error of  $<1\%$ .

## **Chapter 3: Total fluoride concentration in tea leaf products**

### 3.1 Introduction

Fluoride concentration can be an indication of the part of the tea plant used in the manufacture of the product, as there is more fluoride in the mature leaves than the tip of the plant, (Chapter 1, Table 1.2). Total fluoride concentration in dry tea products can range between 2.1 to 1175 mgkg<sup>-1</sup>, according to Yi and Cao (2008), (Table 3.1). Oolong teas appear to contain the lowest fluoride concentrations with brick teas containing the highest. Brick tea is a Chinese speciality tea, manufactured by compressing mature leaves, twigs and berries into a tablet. Use of the mature leaves is reflected by the high fluoride concentrations in the product (Table 3.1) (Chao *et al.* 1995; Cao *et al.* 1998; 2005). Once used as a currency, Brick teas are still consumed, often in regions where endemic skeletal fluorosis exists (Chao *et al.* 1995; Cao *et al.* 1998, 2005; Baskaradoss *et al.* 2008).

A concentrated tea infusion is used to manufacture instant tea, where the insoluble particles are filtered off and the filtrate is evaporated to leave the instant product (Sinija *et al.* 2007). They differ to tea leaf and bagged products as they dissolve completely into the hot water, rather than result from an infusion. With these beverages, a higher fluoride source can be available, Table 3.1 (Whyte *et al.* 2008; Yi and Cao, 2008). As the insoluble particles are removed, all of the fluoride present in the product will be dissolved in to the beverage.

Total fluoride in tea leaf and bagged products is not a reflection on what is actually consumed in a tea infusion beverage, therefore not a direct impact on human health. However, an amount of fluoride from the product is released into a tea infusion; therefore, tea can be considered a source of fluoride (Fung *et al.* 1999; Malinowska *et al.* 2008; Yi and Cao 2008). The potential exposure of fluoride to the human system from consuming tea can be estimated by determining the rate of transfer of

fluoride into the tea infusion (Mehra *et al.* 2013). Total fluoride in tea products is required for this calculation, together with fluoride concentration from the tea infusion (Chapter 4). The percentage rate of fluoride transfer is useful to determine any characteristics of fluoride release within different tea products.

Table 3.1 Range of fluoride content in various teas

**Content removed for copyright reasons**

Table data was extracted from:

Yi J. and Cao J. (2008). Tea and Fluorosis. *Journal of Fluorine Chemistry*. **129** 76-81.

The aim of this chapter is to determine total fluoride in tea products. Towards this aim the following objectives were addressed:

- To evaluate total fluoride concentrations in a range of tea products.
- To identify any significant relationships in fluoride concentration within different types of tea products.

## 3.2 Materials and Methods

### 3.2.1 Materials

Selections of thirty eight tea products were purchased from UK supermarkets, with the exception of two products from India and one from Sri Lanka (Table 3.2). Products included black blends, green blends, Assam, Darjeeling, Oolong and Pu'er teas, either in loose leaf or tea bag form. Teas were classified into the following groups, Pure blends, Oolong/Pu'er, Black blends, Green blends and Economy blends.

Certified reference material (CRM) tea GBW07605 (Institute of Geophysical and Geochemical Exploration, China) was used to determine the accuracy of % fluoride concentration recovery for quality assurance of the data generated.

Table 3.2 Tea brand, variety, country of purchase, date code and blend for the samples analysed

Product	Variety	Country of purchase	Best before date	Blend description
<b><i>Pure blends</i></b>				
Tata leaf	Black	India	Packed Apr-08	Assam, India
Assam leaf	Black	UK	Sep-11	Blend
Dilma Ceylon bags	Black	UK	Mar-12	Sri Lankan
Liptons Darjeeling leaf	Black	India	May-09	India
Lovers Leap Ceylon leaf	Black	Sri Lanka	Not stated	Sri Lankan
Twinings Assam bags	Black	UK	Apr-11	Imported blend
<b><i>Oolong/Pu'er</i></b>				
Luk Yu Oolong bags	Oolong	UK	Dec-12	Not stated
Luk Yu Pu'er bags	Pu'er	UK	Aug-12	Not stated
Oolong leaf	Oolong	UK	Aug-11	Chinese
Pu'er leaf	Black	UK	Not stated	Chinese



Table 3.2 Tea brand, variety, country of purchase, date code and blend for the samples analysed continued...

Product	Variety	Country of purchase	Best before date	Blend description
<b><i>Black Blends</i></b>				
Jacksons bags	Black	UK	Sep-10	Imported blend
PG Tips bags	Black	UK	Jul-09	Not stated
PG Tips decaff bags	Black	UK	Aug-10	Not stated
PG Tips leaf	Black	UK	Aug-09	Not stated
Twinings Everyday bags	Black	UK	Sep-10	Imported blend
Typhoo bags	Black	UK	Oct-09	Not stated
Yorkshire leaf	Black	UK	Oct-11	Assam and African recipe
<b><i>Green blends</i></b>				
Clipper Organic leaf	Green	UK	Jun-11	Blend
Famous Green leaf	Green	UK	Not stated	Not stated
Green Twinings bags	Green	UK	Oct-10	Not stated
PG Tips Green bags	Green	UK	Aug-11	Pure green
Tetley Green bags	Green	UK	May-12	Blend
Xiamen Green bags	Green	UK	Sep-12	Not stated
<b><i>Economy blends</i></b>				
Asda Smartprice bags 1	Black	UK	Jan-11	Not stated
Asda Smartprice bags 2	Black	UK	Apr-12	Not stated
Asda Smartprice bags 3	Black	UK	Jul-12	Not stated
Euroshopper bags	Black	UK	Dec-10	Not stated
Morrisons Value bags 1	Black	UK	Jan-12	Not stated
Morrisons Value bags 2	Black	UK	Aug-11	Not stated
Morrisons Value bags 3	Black	UK	Jan-13	Not stated
Sainsbury Basics bags 1	Black	UK	Aug-12	Not stated
Sainsbury Basics bags 2	Black	UK	Oct-12	Not stated
Sainsbury Basics bags 3	Black	UK	Feb-13	Not stated
Tesco Value bags 1	Black	UK	Mar-11	Not stated
Tesco Value bags 2	Black	UK	Jul-12	Not stated
Tesco Value bags 3	Black	UK	Nov-12	Not stated
Tesco Value bags 4	Black	UK	Jul-13	Not stated
Waitrose Essential bags	Black	UK	Feb-13	Not stated

### **3.2.2 Methods**

#### ***a. Sample preparation and storage***

Samples were milled using a laboratory mill (IKA M20 Labortechnik, Germany), and passed through a <125 µm sieve to obtain a representative sample, as described in Section 2.2.1.2b(ii). After sieving, samples were transferred in to Kraft bags, made of wet strength brown paper. Between samples, a sieve brush was used to clean residue from the mill. A small portion of the next sample was milled, and this was discarded. This step was repeated twice more and the third grind was collected as the sample. In the Kraft bags, samples were dried at 60 °C in an oven for 16 hours and subsequently stored in a desiccator at room temperature, avoiding direct sunlight prior to analysis (Fung *et al.* 2003).

#### ***b. Tea leaf product digestion***

Alkali fusion, using the aqueous potassium hydroxide Method, Section 2.2.1.2 c, was followed where a total of 0.5 g of prepared tea sample was weighed accurately to 4 decimal places into a nickel crucible and 2 ml of 50% (w/v) potassium hydroxide solution added (Metrohm 2008). Thorough mixing was achieved by gently shaking and agitating each crucible. Sample fusion was initiated by placing the crucibles on a hotplate calibrated to 100 °C for 30 minutes. The crucibles were heated further in a muffle furnace set at 300 °C, increasing the temperature by 50 °C every 15 minutes, up to 600 °C, where they remained for a further 30 minutes (Sparks *et al.* 1996). Cooled samples were transferred quantitatively to 50 ml volumetric flasks, made up to volume with deionised water and filtered through 0.45 µm cellulose nitrate filters into 30 ml polystyrene sterile universal containers (Metrohm 2008).

### ***c. Analysis for total fluoride concentration by ion chromatography***

Fluoride concentrations in the tea products were analysed using a Metrohm IC basic 792 with conductivity detector, chromatographic column Metrohm A Supp 5-250 and a Metrohm 4/5 guard column. The eluent was prepared with 3.2 mmol l<sup>-1</sup> sodium carbonate and 1.0 mmol l<sup>-1</sup> sodium hydrogen carbonate, operating with flow rate of 0.7 ml per minute, a run time of 32 minutes and a 20 µl injection volume.

Calibration was carried out every 12 injections, using 1, 2, 3, 4, 5 and 10 mg l<sup>-1</sup> fluoride standards, prepared from 1000 mg l<sup>-1</sup> fluoride standard stock solution (Fisher Scientific). A chromatogram for each sample provided a value of fluoride in mg l<sup>-1</sup>. The following calculation converted fluoride to mg kg<sup>-1</sup>.

$$[\text{F}^-] \text{ mg kg}^{-1} = \frac{[\text{F}^-] \text{ mg l}^{-1} \times \text{dilution factor (ml)}}{\text{Weight of sample (g)}}$$

### **3.2.3 Quality control**

Certified reference material tea GBW07605 was digested with every 10 samples to determine the accuracy of the results. Three replicates of each tea sample and replicate sample blanks were carried out to evaluate precision and to identify possible interferences, respectively.

All reagents were of analytical grade or above and glassware was pre-acid washed with 10% (w/v) nitric acid and triple rinsed with de-ionised water.

### 3.2.4 Data analysis

Statistical analysis of the data was carried out as described, Section 2.2.5, involving Microsoft Excel for descriptive statistics and Students t-tests with IBM SPSS software to determine normality of data and to calculate ANOVA with *post-hoc* Bonferroni multiple comparison tests.

## 3.3 Results

### 3.3.1 Total fluoride determination in tea products

Mean total fluoride concentrations for the individual tea products are shown in Table 3.3. Data ranged from 103 to 839 mgkg<sup>-1</sup>, with the lowest concentration being Liptons Darjeeling in the Pure blend group and the highest concentration was Economy blends, Asda bags 1. The overall mean and SD for the data set as a whole was 383 ± 206 mgkg<sup>-1</sup>, reflecting a wide range in concentration depending upon the tea type and brand.

Within the Black blends, Jacksons bags fluoride concentration of 130 mgkg<sup>-1</sup> was significantly lower than the other tea's in the group ( $p < 0.05$ ), but similar to most of the teas in the Pure blend group. Within the Economy blends, Waitrose Essential bags fluoride concentration was significantly lower than the other Economy teas in the group ( $p < 0.001$ ), but was similar to PG Tips leaf and Twinings Everyday bags in the Black blend group.

Table 3.3 Mean and SD fluoride concentration ( $\text{mgkg}^{-1}$ ) in tea products (n=3)

Product	[F <sup>-</sup> ] concentration
<b><i>Pure blends</i></b>	
Tata leaf	200 $\pm$ 5
Assam leaf	148 $\pm$ 12
Dilma Ceylon bags	115 $\pm$ 6
Liptons Darjeeling leaf	110 $\pm$ 15
Lovers Leap Ceylon leaf	103 $\pm$ 6
Twinings Assam bags	114 $\pm$ 8
<b><i>Oolong/Pu'er</i></b>	
Luk Yu Oolong	202 $\pm$ 10
Luk Yu Pu'er	268 $\pm$ 12
Oolong leaf	222 $\pm$ 6
Pu'er leaf	174 $\pm$ 6
<b><i>Black Blends</i></b>	
Jacksons bags	130 $\pm$ 11
PG Tips bags	382 $\pm$ 5
PG Tips decaff bags	239 $\pm$ 12
PG Tips leaf	325 $\pm$ 9
Twinings Everyday bags	296 $\pm$ 8
Typhoo bags	271 $\pm$ 9
Yorkshire bags	220 $\pm$ 2
<b><i>Green blends</i></b>	
Clipper Organic leaf	393 $\pm$ 6
Famous Green	172 $\pm$ 7
Green Twinings bags	415 $\pm$ 9
PG Tips Green bags	603 $\pm$ 9
Tetley Green bags	387 $\pm$ 7
Xiamen Green	408 $\pm$ 2

Table 3.3 Mean and SD fluoride concentration ( $\text{mgkg}^{-1}$ ) in tea products (n=3), continued...

Product	[F <sup>-</sup> ] concentration
<b><i>Economy blends</i></b>	
Asda Smartprice bags 1	839 $\pm$ 20
Asda Smartprice bags 2	544 $\pm$ 20
Asda Smartprice bags 3	539 $\pm$ 11
Euroshopper bags	671 $\pm$ 6
Morrisons Value bags 1	532 $\pm$ 15
Morrisons Value bags 2	667 $\pm$ 11
Morrisons Value bags3	556 $\pm$ 16
Sainsbury Basics bags 1	388 $\pm$ 21
Sainsbury Basics bags 2	423 $\pm$ 17
Sainsbury Basics bags 3	556 $\pm$ 7
Tesco Value bags 1	814 $\pm$ 7
Tesco Value bags 2	523 $\pm$ 7
Tesco Value bags 3	698 $\pm$ 20
Tesco Value bags 4	592 $\pm$ 18
Waitrose Essential bags	330 $\pm$ 15
<b>Overall mean and SD</b>	<b>383 <math>\pm</math> 206</b>

Figure 3.1 shows the mean fluoride concentrations for the tea groups. Pure blends have the lowest mean fluoride concentration of  $132 \pm 35 \text{ mgkg}^{-1}$  and the Economy blends have the highest at  $578 \pm 140 \text{ mgkg}^{-1}$ . The most significant difference in fluoride was the Economy blends group being higher in fluoride concentration compared to all the other tea groups ( $p < 0.001$ ).

The Green blends group, with a mean concentration of  $397 \pm 129 \text{ mgkg}^{-1}$  were mid-range in respect to the other tea groups. Significant similarities were observed between the mean fluoride concentration of  $266 \pm 77 \text{ mgkg}^{-1}$  for the Black blends group with the Oolong/Pu'er group at  $216 \pm 37 \text{ mgkg}^{-1}$  ( $p > 0.05$ ) and Oolong/Pu'er group with the Pure blends, ( $p > 0.05$ ). The order of mean total fluoride is Economy blends > Green blends > Black blends > Oolong/Pu'er > Pure blends.

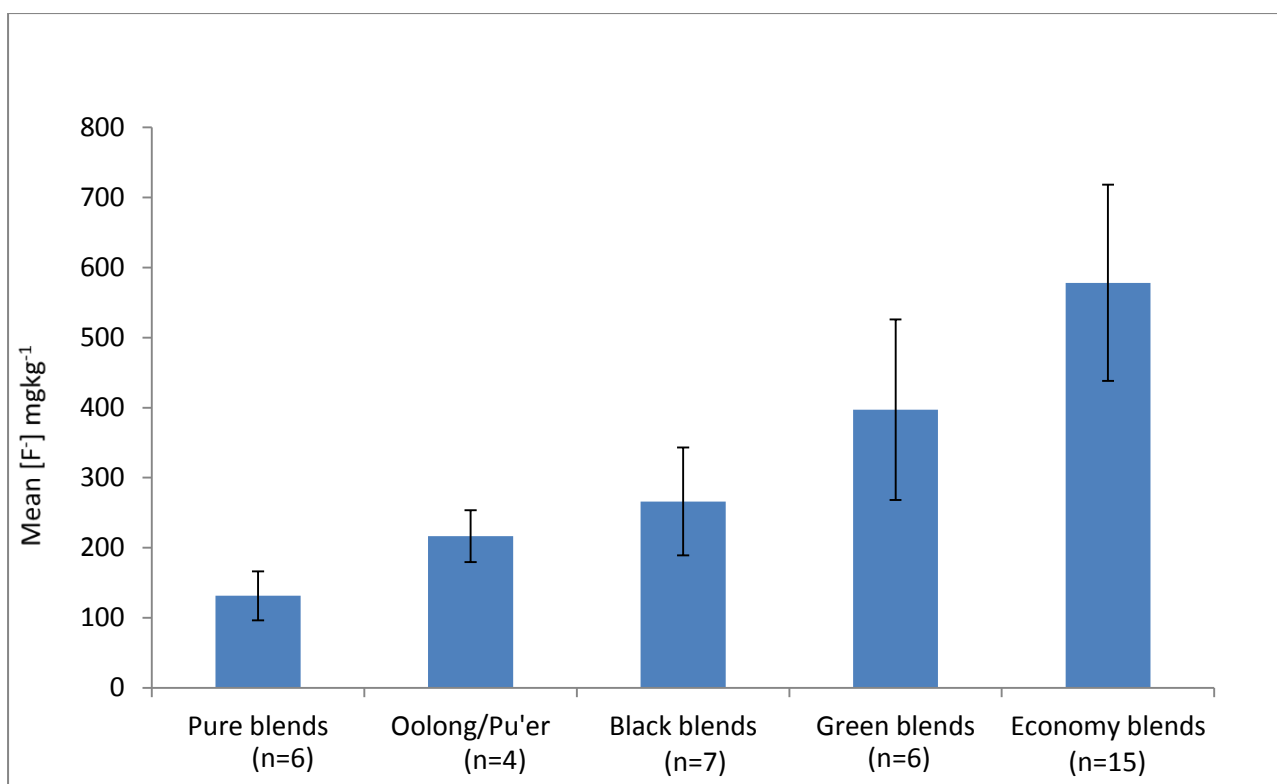


Figure 3.1 Mean fluoride concentration (mgkg<sup>-1</sup>) in the tea groups

### 3.3.2 Quality control

Mean and SD fluoride concentration of the CRM GBW07065 were calculated as  $316 \pm 9$  mgkg<sup>-1</sup>, agreeing with the certified value. This reflected the accuracy of the method (Table 3.4), with the range recovery of 91.8 to 103.4%. The blank samples analysed within the sample batches gave a mean of 0.07 mgkg<sup>-1</sup>, indicating no contamination present.

Table 3.4 Determined fluoride concentrations (mgkg<sup>-1</sup> or mg l<sup>-1</sup>) for CRM and blank

Sample	n	Certified value [F <sup>-</sup> ]	Determined mean and SD [F <sup>-</sup> ]	% Mean recovery
CRM GBW07065	20	$320 \pm 31$ mgkg <sup>-1</sup>	$316 \pm 9$ mgkg <sup>-1</sup>	98.8
Blank	20	n/a	$0.07 \pm 0.1$ mg l <sup>-1</sup>	n/a

## 3.4 Discussion

### 3.4.1 Total fluoride determination in tea products

Total fluoride in the tea products were in harmony with studies carried out by Fung *et al.* (1999), Shu *et al.* (2003) and Malde *et al.* (2006), who reported total fluoride concentrations in the range of 170 to 878 mgkg<sup>-1</sup>, 49 to 708 mgkg<sup>-1</sup> and 100 to 630 mgkg<sup>-1</sup>, respectively.

For the individual tea groups, the Black blend teas, ranged from 130 to 382 mgkg<sup>-1</sup> and were comparable to the data on black teas reported by Fung *et al.* (1999), Yi and Cao (2008) and Koblar *et al.* (2012), 204 to 423 mgkg<sup>-1</sup>, 30 to 385 mgkg<sup>-1</sup>, and 68 to 435 mgkg<sup>-1</sup>, respectively. In the Green blends tea group, total fluoride ranged from 172 and 603 mgkg<sup>-1</sup> and was higher than those reported by Shu *et al.* (2004), 49 to 105 mgkg<sup>-1</sup>. Shu *et al.* (2004) states the green teas in his study were of varying quality and included flower tea. The Green blends from this study did not include flower products and were agreeable with Fung *et al.* (1999) and within the range of those summarised by Yi and Cao (2008) and Koblar *et al.* (2012).

Oolong tea results in this study of 202 and 222 mgkg<sup>-1</sup> were similar to those of Fung *et al.* (1999) and Koblar *et al.* (2012). The Pu'er tea results did not compare, being lower than Fung *et al.* (1999) results, but were similar to the wider range for Pu-er tea of 83 to 235 mgkg<sup>-1</sup> reported by Koblar *et al.* (2012).

Considering Pure, Black and Economy blends are strictly all black blended teas, significant differences were observed from group to group, the order of increasing fluoride was Pure > Black > Economy. Tea varieties from a wide range of countries, such as India, China, Sri Lanka, Jordan and Kenya, are used to create a final tea blend (Heneberry 2006; Pettigrew and Richards 2008; Tea Council 2013d). For instance,



Tetley tea, a popular UK brand, produced under the Tata tea group, use tea from approximately 10,000 tea estates in up to 35 countries (Mehra *et al.* 2007). Variations in tea from different countries used to manufacture the blends could result in differences in fluoride concentrations (Samarasingham 2009; Tea Council 2013b).

Jacksons tea bags were grouped within the Black blends group, but this tea was significantly different to the other tea products in that group, being similar to the Pure blends. The packaging of Jacksons tea describes it as a blend of tea from specific locations of Kenya, Assam and Ceylon and two of these regions are classified as Pure blends in this study. For example, Pure blends Lovers Leap is 100% Ceylon tea and is stated to be sourced from a '*luxurious single estate tea from Sri Lanka*' (Fernando 2002). Therefore, the least number of countries involved in producing a tea blend, may result in lower fluoride concentration in the final product. In addition, fluoride concentration could relate to quality, with lower fluoride concentrations in teas indicating the production is from young buds and high concentrations are the products manufactured from the mature older leaves (Lu *et al.* 2004).

In the Economy blends, Waitrose and two samples of Sainsbury Basics bags were comparable to the Black blends fluoride concentration results (Table 3.3). Waitrose has a reputation for selling high quality produce (Berman *et al.* 2009). Generally, the other Economy blend teas were comparable with brick tea reported by Fung *et al.* (1999) and Shu *et al.* (2003). As previously noted, brick tea is manufactured using mature leaves, which could be the case with the economy brands. Economy tea brands cost much less than the other brands in this study.

In August 2013, Sainsburys Basics bags cost £0.11/100g compared to Jackson's black bags at £1.04/100g and Clipper Organic green leaf at £1.02/100g. The other economy brands from Asda, Morrisons and Tesco were comparably priced to the Sainburys

Basic bags. Waitrose was an exception, costing similar to regular black blends at £0.52/100g.

The four main supermarket leaders in the UK are Asda, Morrisons, Sainsburys and Tesco's, with over 76% of the market, dated January 2012, (BBC 2012), which limits the UK population on choice. This may reflect that the population in the UK who are socio-economically disadvantaged and use economy labelled foods are at more of a risk from higher exposure to fluoride from these tea brands.

Although the tea leaf is not usually consumed, there are practices of using fermented tea as a cooking ingredient (Pruess 2006). In Myanmar, the tea leaf is a delicacy used as part of a salad or as a pickled condiment (Chi and Jackson 2011). Although dependent upon quantity, consumption of the tea leaf would suggest an increased exposure to fluoride from the concentrations reported in this study (Table 3.3). The variation that exists in total fluoride concentration in tea leaf products is dependent upon the brand, type, quality and also price. However, as tea leaves are not generally consumed, it is the concentration of fluoride in the tea infusions prepared from the product, which would relate more to the degree of possible human exposure.

### **3.5 Chapter Summary**

- Total fluoride concentration is significantly variable depending upon the product and UK price. Economy teas in the UK cost much less, but generally have high fluoride concentrations similar to brick teas, prepared from mature leaves.
- Pure blend teas have the lowest fluoride concentrations in their products. These are manufactured from single estate teas, rather than blends of many different countries.

- Consumption of the tea leaf is not recommended because the leaf can contain elevated concentrations of fluoride.

## **Chapter 4: Total fluoride concentration in tea infusions**

## 4.1 Introduction

There is great variability of fluoride present in dry weight samples of tea product and subsequently in tea infusions prepared from their product. Yi and Cao (2008) summarise this variability as range of fluoride concentration from 0 to 33.4mg l<sup>-1</sup> in different tea beverages (Table 4.1).

Table 4.1 Range of fluoride concentrations in various tea beverages

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Table data was extracted from:

Yi J. and Cao J. (2008). Tea and Fluorosis. *Journal of Fluorine Chemistry*. **129** 76-81.

Many studies exist describing fluoride concentrations in tea infusions (Duckworth and Duckworth 1978; Effendi and Wibowo 1984; Smid and Kruger 1985; Schamschula *et al.* 1988; Chan and Koh 1996; Gulati *et al.* 2003; Cao *et al.* 2004; Chandrajith *et al.* 2007; Sofuoglu and Kavcar 2008; Malinowska *et al.* 2008; Yi and Cao 2008; Koblar *et al.* 2012; Quock *et al.* 2012). It is well established that fluoride is present in the beverage consumed and therefore, tea is an important source of fluoride in the diet. Other sources of fluoride include oral hygiene products, wine, processed food and seafood (Chan and Koh 1996; Warren and Levy 1999; Connett and Connett 2001; Fein and Cerklewski 2001; Poureslami *et al.* 2008). Additionally, in some areas, drinking water is artificially fluoridated to approximately 1 mg l<sup>-1</sup>, as in the case of Severn Trent Water supply in the West Midlands, UK (British Fluoridation Society, 2013a). Fluoridation has become a controversial issue, with many groups opposing it (National Health and Medical Research Council 1999; McDonagh *et al.* 2000; Bryson 2006; Curtess 2007).

Although fluoride in the tea plant is complexed with aluminium; in a tea infusion the majority of the fluoride becomes unbound as a free ion ( $F^-$ ) (Horie *et al.* 1992). Ionic fluoride is the most bioavailable form of the fluoride and therefore, tea consumption can act as a vehicle for fluoride dosing in the human diet (Horie *et al.* 1992). This is especially relevant, when considering the average amount of tea consumed by an adult is 1 litre a day (Mehra and Baker 2007). The high gastric acidity of the stomach is advantageous to fluoride absorption as alkalinity decreases its solubility (Cerklewski 1997). In the fasted state almost 100% fluoride absorption takes place, but in the presence of food this is dramatically reduced to 50 - 80% (Ophaug 1990).

The National Academy of Science (NAS), USA recommends the dietary reference intake (DRI) and upper tolerable intake values for fluoride, calculated on body weight, Table 4.2 (NAS 2004). A DRI of 4 mg per day is recommended for adult males and 3 mg per day for adult females, with an upper tolerable intake (UTI) of 10 mg per day (NAS 2004). By determining fluoride concentrations in tea infusions, the DRI values can be considered and compared.

Table 4.2 Fluoride dietary intake mg per day

**Content removed for copyright reasons**

Table data was extracted from:

NAS (2004). *Dietary Reference Intakes (DRI): Recommended Intakes for Individuals, Food and Nutrition Board, Institute of Medicine, National Academies*. [Online]  
Available at:

[http://www.iom.edu/Global/News%20Announcements/~/\\_media/Files/Activity%20Files/Nutrition/DRIs/DRISummaryListing2.ashx](http://www.iom.edu/Global/News%20Announcements/~/_media/Files/Activity%20Files/Nutrition/DRIs/DRISummaryListing2.ashx) [Accessed 10/06/2013].

The aim of this chapter is to determine fluoride concentration in tea infusions prepared from the tea products in Chapter 3. Towards this aim the following objectives were addressed:

- To evaluate fluoride concentration in tea infusions, varying infusion times.
- To determine if particle size has an effect on the rate of fluoride solubility.
- To estimate percentage elemental transfer of fluoride leaching into a tea infusion.
- To estimate the DRI of fluoride from the consumption of tea beverages based on the infusion data generated.

## **4.2 Materials and Methods**

### **4.2.1 Materials**

#### ***a. Un-milled tea***

The thirty eight tea products described in Chapter 3 were analysed (Table 3.2). As described in Section 3.2.1, the teas were classified into the following groups, Pure blends, Oolong/Pu'er, Black blends, Green blends and Economy blends.

#### ***b. Milled tea (<125 $\mu$ m)***

For the milled sample analysis, a smaller selection from those listed in Table 3.2 was chosen (Table 4.3).

Table 4.3 Tea product and classified group for milled to <125 µm analysis

Tea Group	Product
Pure Blend	Tata leaf
Pure Blend	Dilma Ceylon bags
Pure Blend	Twinings Assam bags
Oolong/Pu'er	Pu'er leaf
Black blends	Jacksons bags
Black blends	PG Tips bags
Black blends	PG Tips leaf
Black blends	Twinings Everyday bags
Black blends	Typhoo bags
Green blends	Clipper Organic leaf
Economy blends	Morrisons Value 3

#### 4.2.2 Methods

##### 4.2.2.1 Sample preparation and storage

###### *a. Un-milled tea*

Repeat 'coning and quartering' technique was used on the tea leaves to produce representative samples (McNaught and Wilkinson 1997). Approximately 50 g of un-milled tea was put into a Kraft bag.

###### *b. Milled tea (<125µm)*

Samples were milled using a laboratory mill with repetitive sample clean up, as described in Section 2.2.1.2 b (ii). The milled samples were sieved to <125 µm and placed in Kraft bags.



Both un-milled and milled samples were pre-dried at 60 °C in their Kraft bags for 16 prior to analysis and stored in a desiccator, avoiding direct sunlight at room temperature.

#### **4.2.2.2 Tea leaf infusion preparation**

##### ***a. Un-milled tea***

For the un-milled tea leaf infusion preparation, Section 2.2.1.2 d (i) un-milled, was followed where samples of 2 g were weighed accurately to 4 decimal places into 250 ml Erlenmeyer flasks and 100 ml boiling deionised water added. Flasks were swirled once, capped with foil and incubated between 85 - 90 °C in a water-bath. Infusions were timed at 2, 10 or 30 minutes (Duckworth and Duckworth 1978; Fung *et al.* 1999; Shu *et al.* 2003).

##### ***b. Milled tea (<125µm)***

For the milled tea leaf infusion preparation, Section 2.2.1.2 d (i) milled and sieved to <125 µm, was followed. Tea samples of 0.5 g were accurately weighed to 4 decimal places into 100ml Erlenmeyer flasks and 25 ml boiling deionised water added. Flasks were swirled once, capped with foil and kept between 85 - 90 °C in a water-bath for 2 and 30 minutes respectively.

The tea liquors for both the (a) un-milled and (b) milled tea infusions were filtered through a Whatman No.1 filter paper (Fung *et al.* 1999) and stored in 30 ml polystyrene sterile universal container ready for analysis.

#### **4.2.2.3 Analysis of fluoride concentration by ISE**

Analysis of the prepared tea infusions for fluoride was carried out using ISE as described in Section 2.2.1.2 e (ii), ISE. The ISE instrumentation consisted of a voltmeter with Nico Elit 8221 fluoride electrode and Nico Elit 001 silver chloride reference electrode. TISAB was added to both the samples and standards, at a concentration of 50% (Sparks *et al.* 1996). A sodium fluoride stock solution of 1000  $\text{mg l}^{-1}$  was prepared from Fisher Scientific AR grade sodium fluoride by accurately weighing 0.2211 g into 100 ml de-ionised water. Standards of 0.1, 1.0, 10.0, 100  $\text{mg l}^{-1}$  fluoride (w/v) were prepared from dilutions of the 1000  $\text{mg l}^{-1}$  stock solution. Readings were taken for standards and samples after a stirring period of 1 minute.

The calibration (0.1 to 1000  $\text{mg l}^{-1}$ ) was plotted on a logarithmic scale and the sample results anti-logged, taking the weight and dilution into consideration to give the fluoride concentration in  $\text{mg l}^{-1}$  using Excel™.

#### **4.2.3 Quality control**

Quality control was necessary to ensure accurate measurement of fluoride concentration in the tea infusions and to avoid biased results.

Reagents used throughout the procedure were of analytical grade or purer. All glassware was pre-acid washed, using a 10% nitric acid (w/v) solution bath for at least 12 hours, followed by thorough rinsing with de-ionised water to minimise contamination.

Three replicates for each tea sample, the CRM water NCSZC76304 (LGC Ltd, UK) and a standard of either 3 mg l<sup>-1</sup>, 5 mg l<sup>-1</sup> or 6 mg l<sup>-1</sup> fluoride, prepared from 1000 mg l<sup>-1</sup> standard stock solution (Fisher Scientific) were analysed every 10 samples, together with a sample blank. These steps evaluated the accuracy of the method and to identify any fluoride contamination of the samples.

#### **4.2.4 Data analysis**

Data collected followed a normal distribution, as described in Section 2.2.5; therefore, independent sample t-tests and ANOVA with *post-hoc* Bonferroni multiple comparison tests were used to determine any significant results. Excel was also used to calculate the descriptive statistics such as the mean, standard deviation and coefficient variance.

### **4.3 Results**

#### **4.3.1 Total fluoride concentrations in tea infusions**

##### ***a. Un-milled tea***

Fluoride concentrations in the 2, 10 and 30 minute tea infusions are shown in Table 4.4. Fluoride concentrations in the un-milled tea infusions ranged from 0.4 to 8.0 mg l<sup>-1</sup> at 2 minutes, 0.5 to 8.3 mg l<sup>-1</sup> at 10 minutes and 0.7 to 9.0 mg l<sup>-1</sup> at 30 minute infusions. Overall mean fluoride concentrations were 3.9, 4.5 and 4.9 mg l<sup>-1</sup> for the 2, 10 and 30 minute infusions, respectively. There were no significant differences in fluoride concentrations for the between the 2 and 10 minute infusions or between the 10 and 30 minute infusions ( $p>0.05$ ), but a significant difference was observed between the 2 and 30 minute infusions ( $p\leq0.001$ ).

Table 4.4 Fluoride concentrations in the un-milled tea infusions (mean  $\pm$  SD, n=3)

Product	F <sup>-</sup> (mg l <sup>-1</sup> ) tea infusion Time of brewing (min) n=3		
	2 min	10 min	30 min
<b><i>Pure blends</i></b>			
Tata leaf	2.3 $\pm$ 0.2	2.9 $\pm$ 0.1	3.1 $\pm$ 0.2
Assam leaf	2.5 $\pm$ 0.1	3.1 $\pm$ 0.3	3.3 $\pm$ 0.1
Dilma Ceylon bags	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.3 $\pm$ 0.1
Liptons Darjeeling leaf	0.7 $\pm$ 0.0	1.1 $\pm$ 0.1	1.2 $\pm$ 0.1
Lovers Leap Ceylon leaf	1.4 $\pm$ 0.1	1.6 $\pm$ 0.1	1.7 $\pm$ 0.0
Twinings Assam bags	1.7 $\pm$ 0.0	2.0 $\pm$ 0.3	1.8 $\pm$ 0.1
<b><i>Oolong/Pu'er</i></b>			
Luk Yu Oolong	1.4 $\pm$ 0.1	2.3 $\pm$ 0.1	2.6 $\pm$ 0.1
Luk Yu Pu'er	0.7 $\pm$ 0.0	1.4 $\pm$ 0.0	1.5 $\pm$ 0.1
Oolong leaf	0.7 $\pm$ 0.1	1.3 $\pm$ 0.1	1.4 $\pm$ 0.3
Pu'er leaf	0.4 $\pm$ 0.0	0.9 $\pm$ 0.1	1.2 $\pm$ 0.0
<b><i>Black Blends</i></b>			
Jacksons bags	0.8 $\pm$ 0.0	0.6 $\pm$ 0.1	0.8 $\pm$ 0.1
PG Tips bags	5.0 $\pm$ 0.4	5.2 $\pm$ 0.2	6.5 $\pm$ 0.3
PG Tips decaffeinated bags	5.0 $\pm$ 0.2	5.3 $\pm$ 0.2	5.4 $\pm$ 0.2
PG Tips leaf	3.9 $\pm$ 0.3	5.0 $\pm$ 0.2	5.3 $\pm$ 0.2
Twinings Everyday bags	3.0 $\pm$ 0.2	3.9 $\pm$ 0.1	4.1 $\pm$ 0.1
Typhoo bags	2.8 $\pm$ 0.4	3.3 $\pm$ 0.1	3.6 $\pm$ 0.3
Yorkshire bags	2.5 $\pm$ 0.2	2.8 $\pm$ 0.1	3.2 $\pm$ 0.1
<b><i>Green blends</i></b>			
Clipper Organic leaf	4.3 $\pm$ 0.7	6.2 $\pm$ 0.8	6.4 $\pm$ 0.5
Famous Green	1.6 $\pm$ 0.1	2.3 $\pm$ 0.1	2.2 $\pm$ 0.1
Green Twinings bags	4.4 $\pm$ 0.3	5.8 $\pm$ 0.2	6.0 $\pm$ 0.3
PG Tips Green bags	3.6 $\pm$ 0.1	5.4 $\pm$ 0.6	6.7 $\pm$ 0.3
Tetley Green bags	2.7 $\pm$ 0.3	3.5 $\pm$ 0.2	3.7 $\pm$ 0.1
Xiamen Green	3.9 $\pm$ 0.9	5.1 $\pm$ 0.2	5.6 $\pm$ 0.3

Table 4.4 Fluoride concentrations in the un-milled tea infusions (mean  $\pm$  SD, n=3) continued...

Product	F <sup>-</sup> (mg l <sup>-1</sup> ) tea infusion $\pm$ SD Time of brewing (min) n=3		
	2 min	10 min	30 min
<b><i>Economy blends</i></b>			
Asda Smartprice bags 1	7.1 $\pm$ 0.0	7.5 $\pm$ 0.3	7.7 $\pm$ 0.4
Asda Smartprice bags 2	5.6 $\pm$ 0.6	6.8 $\pm$ 0.3	7.2 $\pm$ 0.3
Asda Smartprice bags 3	6.7 $\pm$ 0.2	6.9 $\pm$ 0.2	6.9 $\pm$ 0.2
Euroshopper bags	6.9 $\pm$ 0.6	7.7 $\pm$ 0.1	8.0 $\pm$ 0.2
Morrisons Value bags 1	6.7 $\pm$ 0.2	8.4 $\pm$ 0.3	8.8 $\pm$ 0.1
Morrisons Value bags 2	6.4 $\pm$ 0.2	8.1 $\pm$ 0.4	8.6 $\pm$ 0.2
Morrisons Value bags3	5.9 $\pm$ 0.3	6.8 $\pm$ 0.1	6.9 $\pm$ 0.3
Sainsbury Basics bags 1	6.1 $\pm$ 0.8	7.2 $\pm$ 0.1	7.4 $\pm$ 0.3
Sainsbury Basics bags 2	4.4 $\pm$ 0.4	5.2 $\pm$ 0.3	6.6 $\pm$ 0.5
Sainsbury Basics bags 3	5.2 $\pm$ 0.1	5.4 $\pm$ 0.3	6.2 $\pm$ 0.1
Tesco Value bags 1	8.0 $\pm$ 0.1	8.3 $\pm$ 0.1	8.9 $\pm$ 0.1
Tesco Value bags 2	5.6 $\pm$ 0.5	5.6 $\pm$ 0.5	6.2 $\pm$ 0.6
Tesco Value bags 3	5.4 $\pm$ 0.4	5.9 $\pm$ 0.2	6.3 $\pm$ 0.3
Tesco Value bags 4	6.3 $\pm$ 0.3	6.6 $\pm$ 0.1	7.3 $\pm$ 0.4
Waitrose Essential bags	3.6 $\pm$ 0.2	3.7 $\pm$ 0.1	3.9 $\pm$ 0.0
<b>Overall Mean and SD</b>	<b>3.9 <math>\pm</math> 2.2</b>	<b>4.5 <math>\pm</math> 2.3</b>	<b>4.9 <math>\pm</math> 2.5</b>

Mean fluoride concentrations for the timed infusions combined into tea groups are presented in Figure 4.1. The Economy blends group have the highest mean fluoride concentration of 6.0, 6.7 and 7.1 mg l<sup>-1</sup> and the Oolong/Pu'er group have the lowest of 0.8, 1.5 and 1.6 mg l<sup>-1</sup>, for the 2, 10 and 30 minute infusions, respectively, with a significant difference in concentration between these two groups ( $p < 0.001$ ). The order of fluoride concentrations in the tea group for the un-milled infusions is Economy blends > Green blends > Black blends > Pure blends > Oolong/Pu'er. There are significant differences in mean fluoride concentrations between all of the tea groups, with the exception of the Black blends with the Green blends ( $p > 0.05$ ).

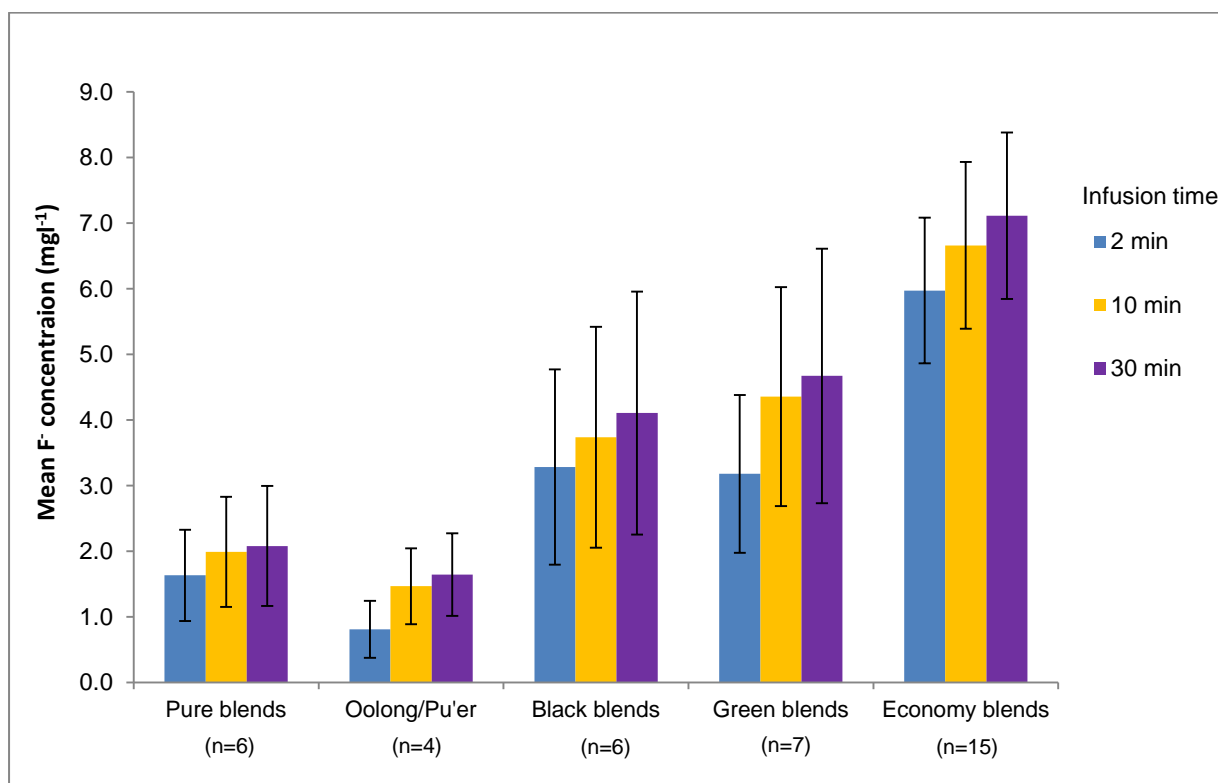


Figure 4.1 Mean fluoride concentrations in the un-milled tea infusion groups (mg l<sup>-1</sup>)

### ***b. Milled tea (<125µm)***

Fluoride concentrations for the milled and sieved to <125 µm particle size infusions are shown in Table 4.5. The infusions ranged from 0.9 to 7.1 mg l<sup>-1</sup> fluoride for the 2 minute infusions, and 1.2 to 7.3 mg l<sup>-1</sup> for the 30 minute infusions. The overall mean and standard deviation was 3.5 ± 2.0 and 3.6 ± 1.9 mg l<sup>-1</sup> for the 2 minute and 30 minute infusions, respectively.

Table 4.5 Fluoride concentrations in the milled (<125  $\mu\text{m}$ ) tea infusion groups ( $\text{mg l}^{-1}$ ) (mean  $\pm$  SD, n=3)

Tea Group	Product	F <sup>-</sup> ( $\text{mg l}^{-1}$ ) tea infusion Time of brewing (min)	
		2	30
Pure blends	Tata leaf	$2.9 \pm 0.2$	$2.9 \pm 0.0$
	Dilma Ceylon bags	$1.2 \pm 0.0$	$1.3 \pm 0.1$
	Twinings Assam bags	$1.6 \pm 0.0$	$1.7 \pm 0.1$
Oolong/Pu'er	Pu'er leaf	$1.0 \pm 0.1$	$1.5 \pm 0.1$
Black blends	Jacksons bags	$1.6 \pm 0.1$	$1.5 \pm 0.1$
	PG Tips bags	$5.8 \pm 0.1$	$5.0 \pm 0.1$
	PG Tips leaf	$5.2 \pm 0.1$	$5.0 \pm 0.4$
	Twinings Everyday bags	$4.2 \pm 0.2$	$4.2 \pm 0.2$
	Typhoo bags	$3.8 \pm 0.2$	$3.8 \pm 0.3$
Green blends	Clipper Organic leaf	$4.6 \pm 0.3$	$5.0 \pm 0.1$
Economy blends	Morrisons Value bags 3	$7.0 \pm 0.1$	$7.2 \pm 0.1$
<b>Overall mean <math>\pm</math> SD</b>		<b><math>3.5 \pm 2.0</math></b>	<b><math>3.6 \pm 1.9</math></b>

Mean fluoride concentrations for the milled and sieved to <125 $\mu\text{m}$  teas are presented in Figure 4.2. At the 2 minute infusion, Pu'er tea leaf had the lowest fluoride concentration of  $1.0 \pm 0.1 \text{ mg l}^{-1}$  and Morrisons Value bags 3 was the highest,  $7.0 \pm 0.1 \text{ mg l}^{-1}$  ( $p>0.001$ ). At 30 minutes, Pure blend Dilma Ceylon bags had the lowest fluoride concentration,  $1.3 \pm 0.1 \text{ mg l}^{-1}$ , with Economy blends Morrisons Value bags 3 having the highest concentration of  $7.2 \text{ mg l}^{-1}$  ( $p>0.001$ ). The order of fluoride concentrations in the tea groups for the milled infusions is Economy blends > Green blends > Black blends > Pure blends > Oolong/Pu'er.

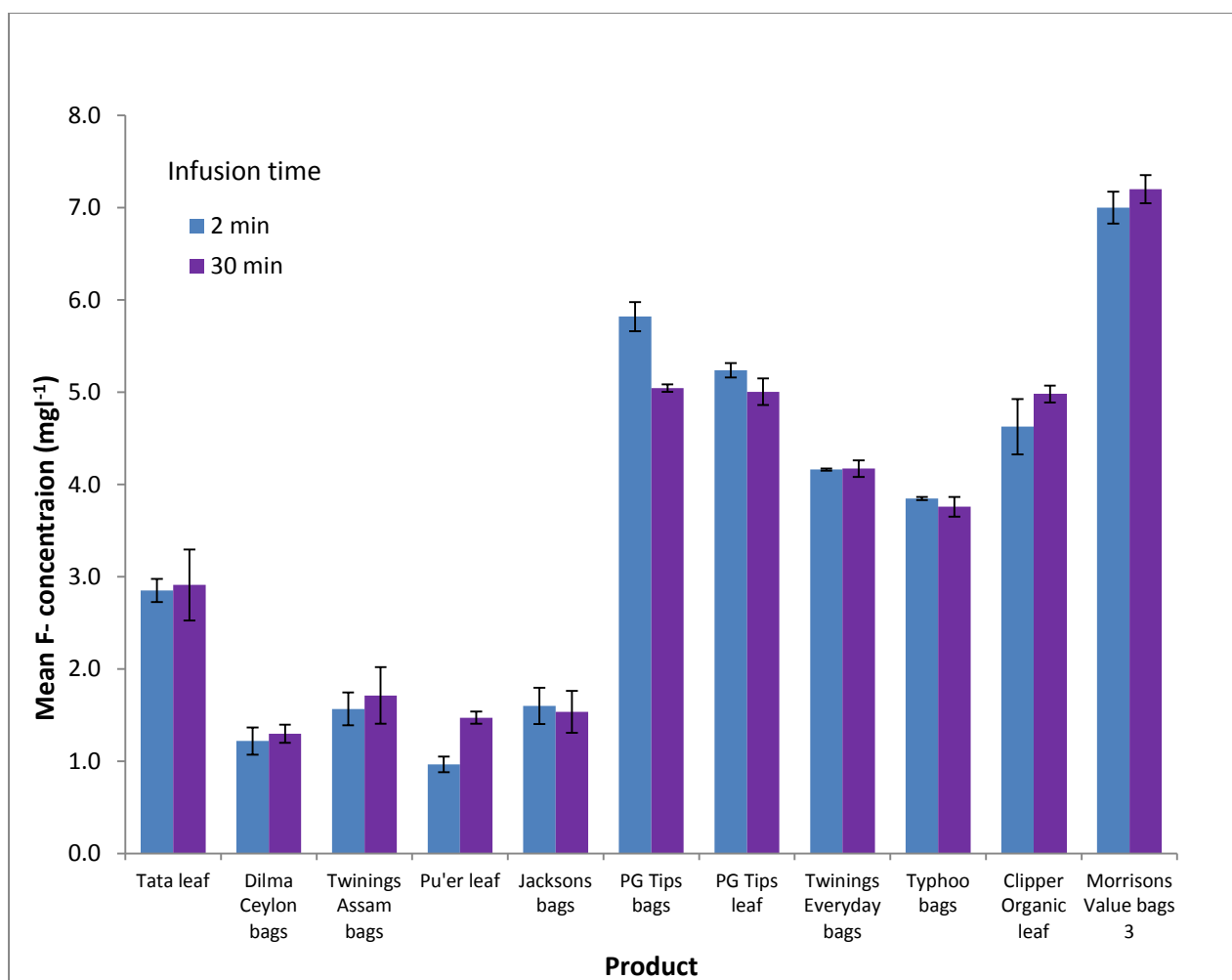


Figure 4.2 Mean fluoride concentrations in the milled tea product infusions (mg l<sup>-1</sup>)

#### 4.3.2 Quality control

As described in Section 2.3.6, precision was calculated using PG Tips black blend tea leaf infusion replicates (n=10) and this gave a percentage coefficient of variance <10%.

Percentage recovery for the CRM NCSZC76304 water was  $90.6 \pm 7.1\%$  (mean  $\pm$  SD) and for the 3, 5 and 6 mg/L standards, recoveries were  $96.3 \pm 3.5\%$ ,  $96.3 \pm 2.1\%$  and  $97.2 \pm 2.6\%$  respectively, indicating the accuracy of the methods.

Fluoride concentration was detected in the sample blank at  $0.03 \pm 0.02$  mg l<sup>-1</sup>, which was deemed as <0.5 mg l<sup>-1</sup> and not taken into account in the sample results.



## 4.4 Discussion

### 4.4.1 Total fluoride concentration in tea infusions

#### *a. Un-milled tea*

Fluoride concentrations in the infusions of the present study are similar to the findings of Fung *et al.* (1999) who reported a range of 0.5 to 7.1 mg l<sup>-1</sup> and Yi and Cao (2008) who presented a wider range of 0.0 to 33.4 mg l<sup>-1</sup>. Quock *et al.* (2012) report a lower range of 0.2 to 5.8 mg l<sup>-1</sup> of fluoride after a 5 minute infusion time. However, the infusions in their study were prepared using 1 g of tea leaf in 100 ml de-ionised water, compared to 2 g in 100 ml adopted in this study, which could compensate for any difference. Also their tea samples included speciality tea, such as Earl Grey, iced blends, orange Pekoe and pomegranate green tea, not included in this study (Quock *et al.* 2012).

A significant increase in fluoride concentration was observed between the 2, 10 and 30 minute infusions ( $p < 0.001$ ), Table 4.4. This indicated an increase in fluoride release with increasing infusion time, agreeing with literature (Duckworth and Duckworth 1978; Effendi and Wibowo 1985; Smid and Kruger 1985; Schamschula *et al.* 1988; Chan and Koh 1996; Gulati *et al.* 2003; Cao *et al.* 2004; Chandrajith *et al.* 2007; Sofuoglu and Kavcar 2008; Malinowska *et al.* 2008; Koblar *et al.* 2012; Quock *et al.* 2012).

Pu'er and Oolong tea fluoride concentrations (Table 4.4) are comparable with those published by Fung *et al.* (1999) and Malinowski *et al.* (2008). Pu'er teas use only the bud and tender leaves of the broad leaf variety of tea (*C. sinensis assamica*) found in southern Yunnan (Reid 2012). Oolong tea is made from tea bushes grown in specific locations, such as Dong Ding Mountain in Taiwan (Reid 2012). Both Oolong and Pu'er

tea varieties are often rolled into long curled leaves, having a large particle size compared to other tea blends and are classified as grade one quality teas (Lu *et al.* 2004). Fluoride release could be slower in the Oolong/Pu'er teas compared to black tea due to the smaller surface area.

The Pure blend teas are comparable with the Ceylon, Assam and Darjeeling teas reported by Malinowska *et al.* (2008), where infusion time was not a significant factor. Similar fluoride release was observed for the 2, 10 and 30 minute infusions for all of the teas within this group ( $p>0.05$ ). This reflects that most of the ionic fluoride will be released into the tea beverage at a 'normal' brewing time of 2 minutes.

Malinowska *et al.* (2008) reported fluoride concentration for a black tea group ranging from 0.5 to 6.1 mg l<sup>-1</sup> after a 10 minute infusion period, which is comparable to the present study. The infusions prepared using Jacksons bags were significantly lower in fluoride concentration compared to the other teas within the Black blend group ( $p<0.05$ ), similar to the tea product (Chapter 3). PG Tips decaff bags were the second highest fluoride concentration tea in the Black blend group, ranging from 4.8 to 5.6 mg l<sup>-1</sup>. According to the study by Chan and Koh (1996), decaffeinated tea products contained twice as much fluoride as caffeinated products.

Malinowska *et al.* (2008) and Koblar *et al.* (2012) reported fluoride concentrations from green tea between 0.8 - 2.1 and 0.6 - 2.4 mg l<sup>-1</sup>, which were markedly lower than the concentrations identified in the present study (2.3 - 6.2 mg l<sup>-1</sup>). Green tea was once a Chinese speciality tea, but recently has become increasingly popular, especially in the UK, since it states to have many health benefits (Imai *et al.* 1997; Liao 2001; Setiawan *et al.* 2001; Venables *et al.* 2008). The range of green tea in the UK is now vast with top brands and supermarket chains producing their own varieties, which could account for possible variations.

Elevated fluoride in brick tea was reported by Fung *et al.* (1999) with concentrations of 4.2 and 7.1 mg l<sup>-1</sup>, which was comparable to the fluoride in the Economy blends analysed in this study. Fung *et al.* (1999) and Cao *et al.* (2001) state that brick tea contains high fluoride as it is the mature leaves used in the manufacturing process, which could also be the case with the Economy blends. Fluoride in the top bud with two leaves can range from 54 - 181 mg l<sup>-1</sup> compared to an over 10 fold increase in concentration in the mature leaves (Shu *et al.* 2003). When tea is harvested, the tea quality is often graded by the part of the plant. An example is Silver Tips or Yin Zhen tea, which only uses the bud without any leaves and is considered an extremely high quality tea, appreciated by connoisseurs (Saber 2010).

Fine plucking is where the bud and two lower leaves are handpicked and coarse plucking is where more leaves are taken (Saber 2010). Coarse plucking is known to produce a lower quality stronger tea (Willson 1999). Machine harvesting may be used rather than hand plucking which is often more cost efficient due to reduced labour costs, however, quality can be affected as selectivity of the tender shoots is lost (Shahony 2010). Economy blends may adopt such coarser methods of harvesting which may be why they cost much less compared to the market leading products.

#### ***b. Milled tea (<125µm)***

For all of the milled <125 µm tea samples, fluoride concentrations were not significantly different between the 2 and 30 minute infusions ( $p < 0.05$ ) with the exception of Pu'er leaf ( $p > 0.01$ ) (Table 4.5). This enhances the theory that particle size can affect the rate of fluoride released into a tea infusion. The smaller the particle size of the tea leaf, the faster the fluoride is released into solution.

When comparing fluoride concentrations of the milled/sieved to <125 µm infusions with the data from the un-milled infusions, a significant difference in fluoride concentration is observed for all of the tea products ( $p < 0.05$ ) at the 2 minute infusion time, except Pure blend Dima Ceyon, Black blend PG Tips bags and Green blend Clipper Organic ( $p > 0.05$ ) (Table 4.5). For the 30 minutes infusions, there was no significant difference between the particle sizes (milled versus un-milled) of the products ( $p > 0.05$ ), except Black blend Jacksons bags and Green blend Clipper Organic. This suggests for most of the products tested, when milled to a smaller particle size, most of fluoride is released faster, after a 2 minute infusion time. If the particle size is larger, as in the purchased product un-milled form, fluoride is released gradually with increasing infusion time. This agrees with the study carried out by Gulati *et al.* (1993) where leaching of fluoride in a tea infusion was found to increase with decreasing particle size.

Consumers of tea would not necessarily change the form of the tea product, i.e. mill before use. However, it was observed from the samples analysed, different brands and tea types do not have a uniform particle size (Appendix 1). Infusing for 30 minutes before consumption is also unlikely, although the use of a teapot may result in longer infusion periods compared to the 'one cup - one tea bag' preparation.

#### **4.4.2 Percentage elemental transfer**

Using the data generated from Chapter 3 (total fluoride in the products), together with fluoride in the infusions data, the percentage of fluoride transferred at 2, 10 and 30 minutes was calculated (Table 4.6).

Table 4.6 Fluoride elemental transfer (%) for different infusion time

Group and product	Elemental transfer % [F <sup>-</sup> ]		
	Infusion time		
	2 min	10 min	30 min
<i>Pure blends</i>			
Tata leaf	58.3	72.9	77.6
Assam leaf	84.7	100.0	100.0
Dilma Ceylon bags	50.4	53.0	57.8
Liptons Darjeeling leaf	32.4	49.7	54.3
Lovers Leap Ceylon leaf	66.0	79.6	83.0
Twinings Assam bags	76.1	87.5	79.2
<i>Oolong/Pu'er</i>			
Luk Yu Oolong	35.5	56.3	63.7
Luk Yu Pu'er	13.0	26.1	27.4
Oolong leaf	15.1	29.5	31.1
Pu'er leaf	12.4	25.6	33.3
<i>Black blends</i>			
Jacksons bags	29.2	24.2	29.2
PG Tips bags	65.1	68.5	84.4
PG Tips decaff bags	100.0	100.0	100.0
PG Tips leaf	59.7	77.4	80.9
Twinings Everyday bags	51.3	65.6	68.3
Typhoo bags	52.0	61.0	66.9
Yorkshire bags	57.4	63.5	73.3
<i>Green blends</i>			
Clipper Organic leaf	54.9	78.9	81.5
Famous Green	47.1	65.4	64.5
Green Twinings bags	53.3	69.4	71.7
PG Tips Green bags	30.1	45.0	55.3
Tetley Green bags	35.3	45.2	47.4
Xiamen Green	47.8	62.2	68.3

Table 4.6 Fluoride elemental transfer (%) for different infusion times continued...

Group and product	Elemental transfer % [F <sup>-</sup> ]		
	Infusion time		
	2 min	10 min	30 min
<i>Economy blends</i>			
Asda Smartprice bags 1	42.6	44.5	46.0
Asda Smartprice bags 2	51.7	62.5	65.9
Asda Smartprice bags 3	62.4	64.0	64.3
Euroshopper bags	51.1	57.1	59.6
Morrisons Value bags 1	63.3	78.7	82.7
Morrisons Value bags 2	48.2	60.5	64.5
Morrisons Value bags 3	52.8	60.8	61.7
Sainsbury Basics bags 1	78.9	92.7	95.3
Sainsbury Basics bags 2	51.7	61.8	78.0
Sainsbury Basics bags 3	46.8	48.6	55.8
Tesco Value bags 1	48.9	50.7	54.4
Tesco Value bags 2	53.3	53.8	59.6
Tesco Value bags 3	38.7	42.3	45.2
Tesco Value bags 4	52.9	55.8	61.3
Waitrose Essential bags	54.5	56.1	59.1

Generally, a wide variation in the percentage of fluoride transferred into the infusion was observed, depending upon tea product rather than tea group. Mostly an increase in the transfer of fluoride to the infusion was observed with increasing infusion time. Both of the Pu'er teas had the least fluoride transferred in the 2 minute infusion, followed by Oolong leaf. As previously mentioned, these tea types have a larger particle size, compared to other tea commodities and the smaller surface area could be the reason why the transfer is much less.

Transfer of fluoride in PG Tips decaffeinated product was the highest with 100% transferred into the 2 minute infusion. Studies by Mehra and Baker (2007) reported considerably higher elemental concentrations of aluminium, copper and manganese in a decaffeinated product compared to non decaffeinated products, but the percentage elemental transfer of these elements was lower. This was not observed

in the present study. However, it could be considered whether or not the method of decaffeination could affect the transfer of fluoride.

The mean percentage elemental transfer of fluoride for the tea groups for 2, 10 and 30 minute infusions is presented in Figure 4.3. Elemental transfer was in the following order:

2 minute infusions: Pure blends > Black blends > Economy blends > Green blends > Oolong/Pu'er.

10 minute infusions: Pure blends > Black blends > Green blends > Economy blends > Oolong/Pu'er.

30 minute infusions: Pure blends > Black blends > Green blends > Economy blends > Oolong/Pu'er.

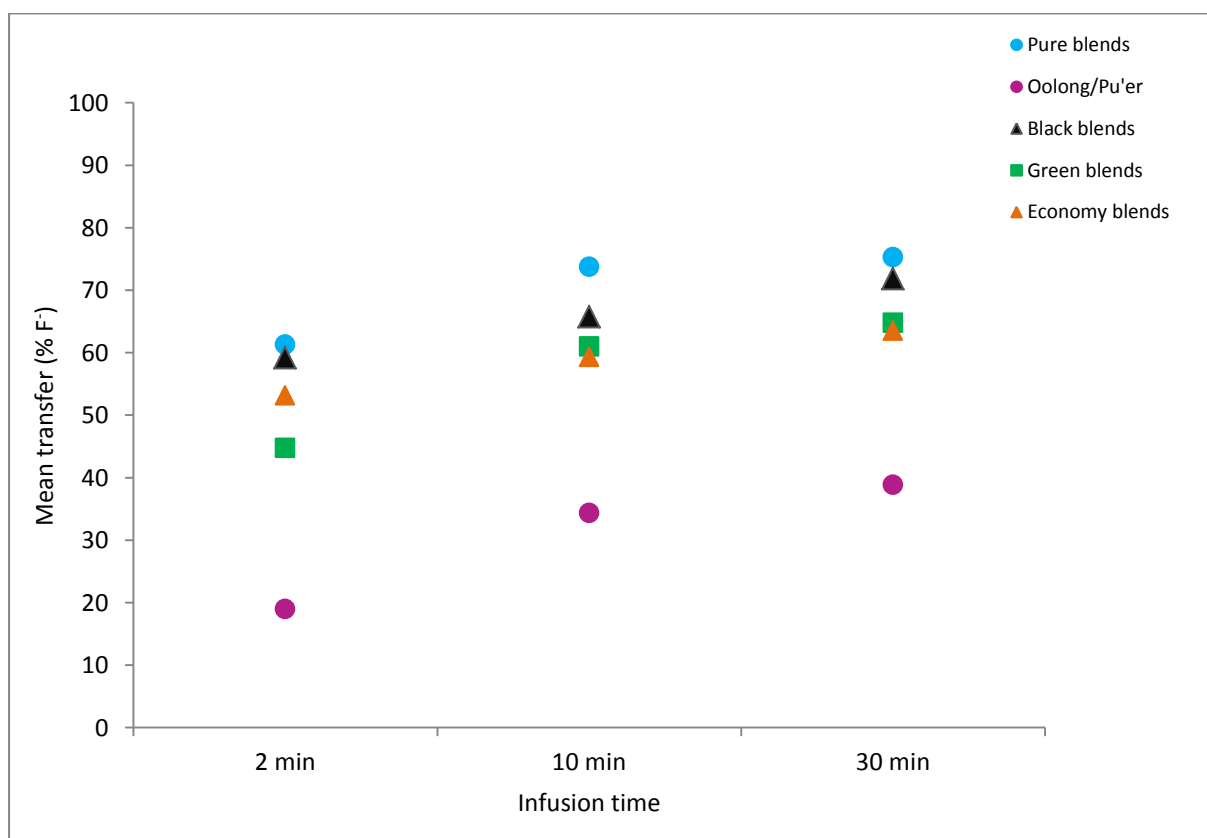


Figure 4.3 Mean percentage transfer for the tea groups (%  $[F^-]$ )

There was no significant difference between transfer ratios of the Green and Economy groups ( $p>0.05$ ) at 10 and 30 minute infusions or between Pure and Black blends at 2 and 30 minutes, showing similar trends in fluoride leaching. Oolong/Puer teas transferred a significantly lower fluoride concentration compared to all the other tea groups ( $p<0.01$ ). This suggests that even after 30 minutes of infusion, > 60% of fluoride still remains in the 'larger' particle sized tea leaves.

#### **4.4.3. Dietary reference intake (DRI)**

The DRI intake of 4 mg a day was used to calculate the percentage of fluoride potentially available for uptake by the human system from consuming 1l of tea using a 2 minute infusion (Mehra and Baker 2007). The 2 minute infusion was also considered typical to preparing tea for human consumption. According to Ophaug (1990), 50 - 80% of fluoride is available for absorption by the human system in the normal state (in the presence of food) and 100% of fluoride is available in the fasted state. Table 4.7 shows the potential fluoride absorption for this study under 'normal' body conditions (in the presence of food) and when the body is in the 'fasted' state using the percentages stated by Ophaug (1990) and using a calculation reported by Mehra and Baker (2007).

Overall, the mean fluoride concentrations for the tea infusions used in the calculations was  $3.0 \pm 2.0 \text{ mg l}^{-1}$  (Table 4.7) and this would provide 42 - 61% of the DRI of fluoride in the normal state and 77% in the fasted state. The Economy blend teas provide 75 - 120% of the daily fluoride in the presence of food and 150% in the fasted state. Oolong/Pu'er teas have the lowest percentage fluoride available of 10 - 16% in non-fasted subjects.



Table 4.7 Mean fluoride (%) available by the normal human system from 1 l tea consumption

Dietary reference intake (DRI) fluoride mg/day (NAS 2004)	Mean fluoride concentration in 2 min infusion mg l <sup>-1</sup>	Mean (%) fluoride DRI from 1 l tea consumption (normal state) 50 – 80% (Ophaug 1990)	Mean (%) fluoride DRI from 1 l tea consumption (fasted state) 100% (Ophaug 1990)
4	Oolong/Pu'er: 0.8	10-16	20
4	Pure blends: 1.6	25-40	50
4	Green blends: 3.2	40-64	80
4	Black blends: 3.3	42-66	83
4	Economy blends: 6.0	75-120	150
<b>4</b>	<b>Mean &amp; SD: 3.0 ± 2.0</b>	<b>42 - 61 ± 25 - 40</b>	<b>77 ± 50</b>

Tea is not the only source of fluoride in the human diet, but it accounts for a high percentage of the daily intake in the presence of food, non-fasted state and even greater in the fasted state (Ophaug 1990). This study used ultra-pure de-ionised water in the infusion preparation. In areas where drinking water is artificially or naturally fluoridated, the fluoride concentration of a tea infusion would increase when prepared using this water (British Fluoridation Society 2013a).

This study indicates fluoride concentrations can exceed the recommended DRI of 4 mg a day (NAS 2004), in certain tea commodities, under the minimal brewing time of 2 minutes (Table 4.7). This is in agreement with Koblar *et al.* (2012) who report that the adequate intake of fluoride from a 70 kg adult consuming five cups of tea daily ranges from 25 - 210% depending upon tea brand and whether the water is fluoridated. According to Trautner and Einwag (1989) the presence of milk reduced fluoride bioavailability by 30%, but with the addition of food this effect was not observed. The reduction in fluoride availability is thought to be caused by

coagulation with the milk (Trautner and Einwag 1989). With 96% of Britons adding milk to their tea, the availability of fluoride from tea could be reduced. For the Economy blends, the addition of milk would reduce the daily fluoride to 52.5 - 84%, but in the presence of food would still account for over 100% in the fasted state.

People who drink excessive amount of tea may not realise the health implications. Although symptoms of excess fluoride are mostly endemic in China, India and Africa (Malde *et al.* 2003; Cao *et al.* 2005; Baskaradoss *et al.* 2008), Joshi *et al.* (2011) reported a 53 year old woman in the UK who drank 1.4 l of tea a day; hence, ingesting approximately 10.9 mg of fluoride a day. This exceeded the stated DRI of 4 mg a day (NAS 2004) by almost 3 fold; however, the woman also used oral hygiene products in excess. Radiographs showed osteosclerosis of the spine and pelvis and an increase in bone density, typical of skeletal fluorosis. Izuora *et al.* (2011) reported a 48 year old patient in the US with severe joint and bone pain. Since the age of 12, the patient had consumed at least 3.8 l of tea daily. The brand of tea was described as, “the least expensive ‘store brand’” and contained  $3.9 \text{ mg l}^{-1}$  of fluoride. A lateral radiograph of the spine indicated crippling skeletal fluorosis from severe calcification, osteoporosis and increased bone density. Treatment was provided by avoiding all fluoride in the diet and administering vitamin D and calcium, however side effects can also include kidney stones and hypercalciuria (Izuora *et al.* 2011).

Considering these case studies, fluoride concentrations found in the present study could lead to detrimental health conditions in a human, especially if consuming in excess of one litre of the Economy branded tea per day. In contrast, if a Pure blend or an Oolong/Pu'er brand of tea is consumed, the percentage of the DRI of fluoride is only up to 40% (Table 4.7).

## 4.5 Chapter summary

- Fluoride concentrations are significantly variable in tea infusions depending upon the type of tea product. Similar to total fluoride concentration in the products (Chapter 3), the order of fluoride concentrations showed Economy teas containing the most and Oolong/Pu'er teas had the lowest concentrations.
- For the individual tea groups, the mean fluoride concentration in a 2 minute infusion contained significantly lower fluoride compared to longer infusion times of 10 and 30 minutes. Most of the fluoride is released into a tea infusion after 10 minutes.
- Particle size affects the rate of fluoride transfer. If the tea is milled to <125 µm particle size, the solubility of the fluoride is faster.
- Percentage elemental fluoride transfer increased with infusion time, therefore fluoride leaching from the tea product to the infusion is dependent upon time. Oolong/Pu'er teas have the lowest transfer rate with a mean of <60% of fluoride remaining in the tea leaves.
- Fluoride is available for absorption by the human system and can partly or completely fulfil the recommended DRI of 4 mg/day for an adult. Detrimental health effects could occur if an adult consumes one litre of tea daily, especially when prepared using an UK supermarket Economy brand. However, if consuming other tea varieties, such as Oolong/Pu'er, Assam, Ceylon or Darjeeling, then exposure to fluoride is significantly lower.

## **Chapter 5: *In vitro* bioaccessibility of fluoride in tea infusions.**

## 5.1 Introduction

Depending upon target receptors and environmental media, there are different definitions for environmental bioavailability and human bioavailability, which can confuse the definition of bioaccessibility (Semple *et al.* 2004). In environmental science, Alexander *et al.* (1997) defined bioavailability in the context of chemical pollutants to the environment rather than nutrient effect on human health. Ruby *et al.* (1996) reports bioavailability as the amount of a compound removed from soil, which can be transferred to the circulatory system, in association with environmental exposure. In human health sciences, such as nutritional science, bioavailability is the proportion of nutrient or bioactive compound that is digested and used or stored for physiological functions (Benito and Miller 1998). In pharmacology, bioavailability is a measurement of the rate and extent to which a drug reaches the systemic circulation (Fernandez-Garcia *et al.* 2009). In general, the term bioavailability includes availability for absorption, metabolism, distribution in the body and bioactivity (Fernandez-Garcia *et al.* 2009). For the purpose of this study, the Schumann *et al.* (1997) definition of bioavailability will be referred to, which is the fraction of an oral dose of substances from a certain preparation that reaches the circulatory system (blood).

Bioaccessibility is defined as the fraction of an oral dose of a substance that is soluble in the gastric environment and available for absorption (Paustenbach 2000). However, not all of the bioaccessibility fraction may actually be absorbed into the circulatory system; hence, bioavailability is a fraction of the bioaccessibility total (Wragg and Cave 2002). Figure 5.1 depicts the bioaccessibility fractions involved in oral exposure, where  $F_B$  is the fraction mobilised from the matrix into the digestive fluids (Ruby *et al.* 1992). In the context of this study,  $F_B$  would represent the concentration of fluoride released from the matrix tea, in the infusion.  $F_A$  is the fraction of fluoride bioaccessibility from the lumen to the intestinal epithelium and in

the portal vein; this may be metabolized, otherwise excretion can occur (Oomen *et al.* 2003).  $F_H$  is the fraction that is not metabolized by the liver, so it can enter the human systematic circulation. The oral bioavailability fraction,  $F$  is the concentration of fluoride available to the human systematic circulation (Oomen *et al.* 2003). The bioavailability fraction will always be less than or equal to the bioaccessibility fraction (Wragg and Cave 2002).

Figure 5.1 Oral bioaccessibility and bioavailability

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Figure data was extracted from:

Oomen A. G., Rompelberg C. J. M., Bruil M. A., Dobbe C. J. G., Pereboom D. P. K. H. and Sips A. J. A. M. (2003). Development of an *in vitro* digestion model for estimating the bioaccessibility of soil constituents. *Archives of Environmental Contamination and Toxicology*. **44** 281-287, (Figure 1).

Experiments to estimate oral bioaccessibility are also known as physiologically based extraction tests (PBET) or *in vitro* gastrointestinal extractions (Ruby *et al.* 1996). For PBET, gut conditions (stomach and intestine) are simulated in a bench top procedure to ascertain the amount of ingested chemicals dissolved and thus available for crossing the intestinal membrane human (Dean 2006). Temperature, peristaltic movement, body fluids and estimated times of digestion are mimicked in the way the natural human body would function (Oomen *et al.* 2003). To date this test has successfully been used to estimate persistent organic pollutants (POPs), metals and metalloids mostly from soil ingestion (Lock and Bender 1980; Crews *et al.* 1983; Ruby *et al.* 1996; Williams *et al.* 1998; Rodriguez *et al.* 1999; Smith *et al.* 2000; Lopez *et al.* 2002; Oomen *et al.* 2003; Dean and Ma 2007). The halogens (especially fluoride) have had little attention, although Saikat (2004) studied iodine and reported its limited bioaccessibility from soil. Fukushima and Chatt (2012) reported bioaccessible iodide levels to be between 72 – 98% when the lower parts of sea mustard plants are consumed.

With fluoride concentrations in tea infusions of up to 8.0 mg l<sup>-1</sup> from a 2 minute infusion (Chapter 4), tea can be considered a major source of fluoride in the diet. Although the concentrations reported are from the tea infusions, what is available for absorption by the human body is not known. To date, a few *in vivo* studies concerning fluoride have been reported, mostly using animals such as rats (Trautner and Siebert 1986; Cerklewski 1992). These experiments are complex, time consuming and require ethical approval (Wragg and Cave 2002). Developing a simple *in vitro* extraction method, applicable to fluoride can indicate concentrations available for absorption from the tea infusion. The method should also be inexpensive and can overcome the issues of *in vivo* testing. Importantly results should highlight whether there is a possibility of exceeding the optimum fluoride concentration recommended for humans from tea drinking. This would be a novel approach as an *in vitro* method for fluoride in tea infusions has not been attempted.

The aim of this chapter was to develop an *in vitro* method to estimate the bioaccessibility of fluoride from tea consumption. Towards this aim the following objectives were addressed:

- To develop an *in vitro* extraction method to estimate the bioaccessibility of fluoride in a range of tea infusions based on PBET and oral bioavailability literature and available instrumentation.
- To critically assess the validation of data using quality assurance for the fluoride concentrations determined.
- To ascertain absorption in different physiological state i.e. fasting and in the presence of food with the addition of milk and saliva to the infusions, to observe the changes in fluoride absorption.



## **5.2. Materials and Methods**

### **5.2.1 Materials**

Three UK teas were selected from the total fluoride in the products and infusions tests, (Chapters 3 and 4, Table 3.2). Pure blend Twinings Assam bags, Black blend PG Tips leaf and Economy blend Morrisson's Value bags 2 were chosen to represent low, medium and high fluoride concentration teas from the different groups, respectively. Certified reference material water, NCSZC76304 (LGC Ltd, UK) was used to determine the accuracy of the fluoride determination in a liquid matrix.

All reagents were of analytical grade, unless stated otherwise, with the exception of the enzymes (porcine extracted). Glassware was pre-acid soaked in 10% (w/v) nitric acid and triple rinsed in de-ionised water prior to analysis

### **5.2.2 Methods**

#### **a. Sample preparation and storage**

Tea sample infusions were prepared as described in Chapter 2, Section 2.2.1.2 d (i) *Un-milled* samples of 2 g were weighed accurately to 4 decimal places, into 250 ml Erlenmeyer flasks and 100 ml boiling deionised water added. Flasks were swirled once, capped with foil and incubated between 85 - 90 °C in a water-bath. Infusions were brewed for a 2 minute period (Duckworth and Duckworth 1978; Fung *et al.* 1999; Shu *et al.* 2003). The infusions were filtered through a Whatman No.1 filter paper (Fung *et al.* 1999) and stored in capped 100 ml polyethylene bottles, allowed to cool ready for immediate analysis.

## b. Development of a method for assessing bioaccessibility of fluoride from tea infusions

Three methods following Powell *et al.* (1998), Bermejo *et al.* (2002) and Intawongse and Dean (2008) were tested, referred to as A, B and C, respectively. These methods were selected as they were originally carried out on a range of metal ions in food and beverages, and hence being nutritionally based. Due to equipment limitations, methods required some minor adaptations to suit available instrumentation and reagents.

The cooled tea infusions were measured initially for fluoride concentration using the ISE instrumentation set up described in Chapter 2, Section 2.2.1.2 e (ii). A separate aliquot from each tea infusion was taken to undergo *in vitro* digestion in a 150 ml Erlenmeyer flask. Incubations were carried out using a Labnet Shaker Control System 311 DS. After each digestion, a quantified aliquot was taken and centrifuged at room temperature using a Hettich Rotina 46 centrifuge and the supernatant analysed for fluoride using ISE, Section 2.2.1.2 e (ii). A general order for the structure of the *in vitro* extractions and analyses to be performed is summarised in Figure 5. 2.

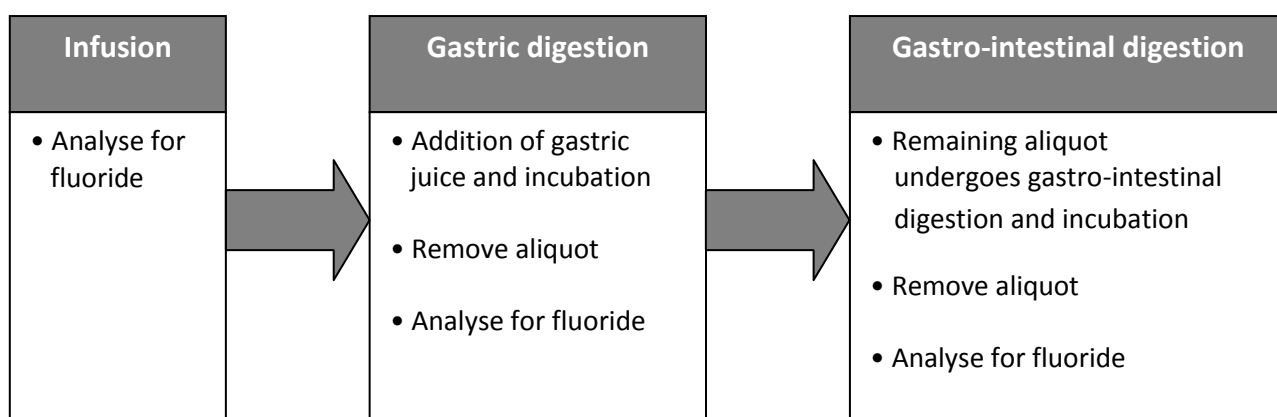


Figure 5.2 Development of an *in vitro* extraction method for fluoride in tea infusions

#### **i. Method A (Powell *et al.* 1998)**

Powell *et al.* (1998) methodology investigated the *in vitro* availability of manganese and trace metals, but not fluoride, from digested tea. Method A was based on this with some minor modifications. Powell *et al.* (1998) describes using human gastric juice and after the gastric digestions were completed, the 'incubates' were centrifuged through ultrafilters. Ultrafilters allow the separation of high molecular weight fractions from the low molecular weights differentiating the analytes available for absorption. The gastric juice composition used in this study was prepared with a simulated juice as in Mounicou *et al.* (2002) and the use of ultrafilters was omitted because of lack of availability. Method A used ISE as the instrumentation instead of inductively coupled plasma optical emission spectroscopy (ICP-OES) as described by Powell *et al.* (1998).

#### **ii. Method B (Intawongse and Dean 2008)**

Intawongse and Dean (2008) used a physiologically-based extraction test for metal ions such as cadmium, copper, iron, manganese and zinc and their bioaccessibility from vegetables. This is a well documented method with many studies in literature citing these experimental conditions (Cui and Chen 2010; Gbefe *et al.* 2011; Karadas and Kara 2011; Intawongse *et al.* 2012; Wragg and Cave 2012). Method B followed the gastric and gastro-intestinal juice constituents and conditions, but the detection of fluoride involved using the ISE rather than inductively coupled plasma mass spectroscopy (ICP-MS).

### **iii. Method C (Bermejo *et al.* 2002)**

Bermejo *et al.* (2002) describe an *in vitro* method for iron and zinc in infant milk formulas and human breast milk. This method involved digesting a liquid rather than a solid matrix and was chosen for that reason. The use of flame atomic absorption spectrometry was used by Bermejo *et al.* (2002), but replaced with ISE in Method C. Centrifuge speed was modified as the centrifuge used was incapable of operating at the speed reported by Bermejo *et al.* (2002) and the temperature could not be set at zero; therefore, it was operated at room temperature.

Gastric and gastro-intestinal juice compositions and incubation times for Methods A, B and C are described and summarised in Table 5.1.

### **c. Bioaccessibility assessment**

Without the availability of a certified reference material with human/animal bioavailability/bioaccessibility data (as none was available) to validate Methods A, B or C, a decision on the method to be developed was based on results from the quality control and the precision of the data generated from the tea products tested. Method C was the method taken forward for subsequent studies (Bermejo *et al.* 2002) as this method gave the most consistent results (Section 5.3.1). As an extension to Method C, further studies looked at any effect from the addition of milk and the addition of saliva. Furthermore, the tea leaf precipitate after filtering the initial tea infusion was dried and digested and also a sample of dried tea leaf product underwent the digestion process.

Table 5.1 Digestive juice compositions and digest periods for Methods A, B and C

Method	A (Powell <i>et al.</i> 1998)	B (Intawongse and Dean 2008)	C (Bermejo <i>et al.</i> 2002)
Gastric juice composition and conditions	10 ml of 10 mgml <sup>-1</sup> pepsin in 150 mMol sodium chloride at pH 2.5 with conc. hydrochloric acid added to 10 ml infusion.	30 ml of 1.25 mgml <sup>-1</sup> pepsin, 2.8 mMol sodium malate, 2.34 mMol sodium citrate, 0.42 ulml <sup>-1</sup> DL-lactic acid (≥85%) at pH 2.5 with conc. hydrochloric acid added to 30 ml infusion.	1 ml of 2 mgml <sup>-1</sup> pepsin in 0.1 M hydrochloric acid added to 30ml infusion; adjust to pH 2 with conc. hydrochloric acid.  Cool in ice bath to stop enzymatic activity.
Gastric Incubation	Incubate at 37 °C and shake at 130 rpm for 1 hour.  Remove 10 ml and centrifuge at 4000 rpm for 10 minutes.  Analyse supernatant for fluoride.	Incubate at 37 °C and shake at 130 rpm for 1 hour.  Remove 30 ml and centrifuge at 4000 rpm for 10 minutes.  Analyse supernatant for fluoride.	Incubate at 37 °C and shake at 130 rpm for 60 minutes.  Remove 15 ml and centrifuge at 4000 rpm for 10 minutes.  Analyse supernatant for fluoride.
Gastro-intestinal composition and conditions	Remaining 10 ml is adjusted to pH 6.5 with saturated sodium hydrogen carbonate.	Remaining 30 ml is adjusted to pH 7 with saturated sodium hydrogen carbonate with addition of 52.5 mg bile salt (microbiological grade) and 15 mg pancreatin.	Remaining 16 ml is adjusted to pH 7 with saturated sodium hydrogen carbonate with addition of 0.15 mgml <sup>-1</sup> pancreatin.
Gastro-intestinal Incubation	Incubate at 37 °C and shake at 130 rpm for 24 hours.  Centrifuge at 4000 rpm for 10 minutes.  Analyse for fluoride.	Incubate at 37 °C and shake at 130 rpm for 4 hours.  Centrifuge at 4000 rpm for 10 minutes.  Analyse for fluoride.	Incubate at 37 °C and shake at 130 rpm for 60 minutes.  Cool in ice bath to stop enzymatic activity.  Centrifuge at 4000 rpm for 10 minutes.  Analyse for fluoride.

## **i. Bioaccessibility of fluoride in tea infusions and tea leaf precipitate**

### ***Infusions***

Infusions were prepared as described in Section 5.2.2a, but the tea leaf precipitate left on the filter paper was also retained. This precipitate was transferred onto a watch glass, dried in an air oven for 16 hours at 60 °C and stored in a Kraft bag. Fluoride concentration was determined in the initial infusion by ISE with instrument conditions described in Section 2.2.1.2 e (ii).

### ***Gastric fraction***

A 30 ml aliquot of infusion was adjusted to pH 2 with concentrated hydrochloric acid in a 150 ml Erlenmeyer flask and 1 ml of artificial gastric juice added, prepared from 2 mgml<sup>-1</sup> pepsin, in 0.1 M hydrochloric acid (Bermejo *et al.* 2002). The flask was sealed using laboratory film and incubated at 37°C, shaking at 130 rpm for 60 minutes. After the timed period, the flask was cooled in an ice bath to stop enzymatic activity (Bermejo *et al.* 2002). A 15 ml aliquot was transferred quantitatively, into a centrifuge tube and rotated at 4000 rpm for 10 minutes. The supernatant was removed, labelled as the gastric fraction and analysed for fluoride concentration.

### ***Gastro-intestinal fraction***

The remaining content in the 150 ml Erlenmeyer flask was neutralised to pH 7.0 using saturated sodium hydrogen carbonate solution and 1 ml of gastro-intestinal juice consisting of 0.1 mgml<sup>-1</sup> pancreatin in 0.1 M sodium hydrogen carbonate added (Bermejo *et al.* 2002). The flask was resealed and incubated for a further 60 minutes at 37°C, 130 rpm, after it was cooled in an ice bath (Bermejo *et al.* 2002). This fraction was centrifuged at 4000 rpm for 10 minutes and the supernatant removed, labelled as the gastro-intestinal fraction and analysed for fluoride concentration.

### ***Tea leaf precipitate***

The tea leaf precipitate was weighed accurately, 1 g into a 100 ml Erlenmeyer flask containing 30 ml of de-ionised water. Artificial gastric juice was added and the method steps continued as described above, Section 5.2.2 c, to obtain the gastric fraction and gastro-intestinal fraction for the analysis of fluoride concentration.

### **ii. Bioaccessibility of fluoride in tea infusions with the addition of saliva**

Artificial saliva was prepared by accurately weighing 0.521 g sodium hydrogen carbonate, 0.088 g sodium chloride, 0.048 g potassium chloride, 0.044 g calcium chloride dehydrate, 0.104 g potassium hydrogen phosphate, 0.416 g mucin and 1.300 g  $\alpha$ -amylase into a 100 ml volumetric flask, making up to the mark with de-ionised water (Poinot *et al.* 2009). To an aliquot of 30 ml tea infusion in a 150 ml Erlenmeyer flask, 3 ml of artificial saliva was added, followed by 1 ml of artificial gastric juice. The digestions were carried out as described in Section 5.2.2 c to obtain both gastric and gastro-intestinal fractions and these were analysed for fluoride concentration.

### **iii. Bioaccessibility of fluoride in tea infusions with the addition of milk**

Semi-skimmed pasteurised cow's milk, 3 ml, was added to 30 ml of tea infusion into a 150 ml Erlenmeyer flask, followed by the digestion stages and analysis described in Section 5.2.2c.

### **iv. Bioaccessibility of fluoride in the tea leaf product**

Pre-dried tea leaf product stored and prepared as described in Section 4.2.2.1, was accurately weighed, 1 g, into a 150 ml Erlenmeyer flask containing 30 ml of de-ionised water. Digestion and analysis were carried out as described in Section 5.2.2 c.

### **5.2.3 Quality control**

For the development of the method, certified reference material NCSZC76304 (water) and a blank were used to determine the ISE performance and any contamination. A standard of 5 mg l<sup>-1</sup> of fluoride in de-ionised water underwent the digestions with the tea infusions to determine percentage fluoride recovery of the method. The tea infusions were prepared as four replicates for the initial method development.

For the bioaccessibility assessment, five or more replicate analyses were used to determine the repeatability. A sample blank and a 5 mg l<sup>-1</sup> fluoride standard were included with every batch of samples, to undergo the same digestion conditions as the tea infusions. For the instrumentation, certified reference material NCSZC76304 (fluoride in water), a blank and a 6 mg l<sup>-1</sup> fluoride standard determined the accuracy and identified any source of contamination.

### **5.2.4 Data analysis**

Data was normally distributed as described in Section 2.2.5 and statistical analysis using Microsoft Excel was used for the calculation of means, standard deviations and Students t-test. IBM SPSS software was used for ANOVA univariate analysis of means with Bonferroni multiple comparisons to determine any significant results.

## **5.3 Results**

### **5.3.1 Methods A, B and C**

The overall mean results for the three methods employed are shown in Table 5.2. Fluoride concentrations in the infusion, gastric and gastro-intestinal fractions for



Method A had the widest range for all the tea products, from 1.5 - 8.3 mg l<sup>-1</sup>, followed by similar ranges for both Methods B and Method C at 1.4 - 7.3 mg l<sup>-1</sup> and 1.4 - 7.0 mg l<sup>-1</sup>, respectively. Twinings Assam bags from the Pure blend group contained the lowest fluoride concentrations in the infusion and digest fractions with Morrisons Value bags 2, from the Economy blends group containing the highest fluoride concentrations and PG Tips leaf being mid range. Results for the tea infusions were in agreement with results shown previously in Chapters 3 and 4.

For Method A, significant differences in fluoride concentrations were not found between the infusions, gastric digestion or gastrointestinal fractions (n=4) for any of the tea products ( $p>0.05$ ). Method B and C gave similar statistical results ( $p>0.05$ ). This indicated the three methods concurred similar fluoride concentrations in the initial infusions and digest fractions for all three tea products. Therefore, the bioaccessibility of fluoride is similar through the simulated digestion for the three methods tested.

Fluoride concentrations in the infusions, gastric and gastrointestinal digests were compared against each method, A, B or C. Method A against Method B showed no statistical difference with any of the tea products ( $p>0.05$ ). Method A against Method C was significantly different for PG Tips leaf in the all digest fractions ( $p<0.01$ ) and for Morrisons Value bags 2 but only in the gastric fraction ( $p<0.05$ ). Method B versus Method C also showed a significant difference in fluoride concentration in the gastrointestinal fraction for PG Tips leaf ( $p<0.05$ ). Fluoride concentrations in Twinings Assam bags were not significantly different between the methods in any of the fractions ( $p>0.05$ ).

Table 5.2 Mean fluoride concentration [ $F^-$ ]  $mg\ l^{-1}$  in the digest fractions for Methods A, B and C (mean  $\pm$  SD, n=4)

Sample and Method	Infusion and digest fraction [ $F^-$ ] $mg\ l^{-1}$		
	Infusion	Gastric	Gastro-intestinal
<b>Twinings Assam bags</b>			
Method A	1.6 $\pm$ 0.1	1.8 $\pm$ 0.1	1.7 $\pm$ 0.2
Method B	1.6 $\pm$ 0.1	1.6 $\pm$ 0.1	1.5 $\pm$ 0.1
Method C	1.8 $\pm$ 0.3	1.6 $\pm$ 0.2	1.7 $\pm$ 0.2
<b>PG Tips leaf</b>			
Method A	4.4 $\pm$ 0.2	4.5 $\pm$ 0.1	4.5 $\pm$ 0.3
Method B	4.2 $\pm$ 0.5	4.1 $\pm$ 0.5	4.1 $\pm$ 0.3
Method C	3.7 $\pm$ 0.3	3.4 $\pm$ 0.3	3.6 $\pm$ 0.2
<b>Morrisons Value bags 2</b>			
Method A	7.1 $\pm$ 0.3	7.6 $\pm$ 0.6	7.5 $\pm$ 0.8
Method B	6.8 $\pm$ 0.5	6.9 $\pm$ 0.3	6.6 $\pm$ 0.5
Method C	6.5 $\pm$ 0.5	6.4 $\pm$ 0.6	6.7 $\pm$ 0.3

The percentage of fluoride bioaccessibility in the digest fractions was calculated against the mean fluoride concentration determined in the initial infusion, Figure 5.3. Method A showed over 100% bioaccessibility of fluoride in all fractions for the three tea products.

Using Method B, fluoride bioaccessibility decreased from the gastric digestion to the gastrointestinal fraction for Twinings Assam, 98.5 - 93.8% and Morrisons Value bags 2, over 100% to 97.0%, but increased with the digestions for PG Tips leaf, 96.4 - 97.0%.

Method C produced more consistent results, with fluoride bioaccessibility ranging from 92.9 - 98.1% in the gastric fraction and 97.1 - >100% in the gastrointestinal

fraction. All tea products had lower percentage fluoride bioaccessibility in the gastric fractions compared to the gastrointestinal fractions. Overall, mean standard deviations for the percentage fluoride bioaccessibility for the methods were in the order of Method A > Method B > Method C.

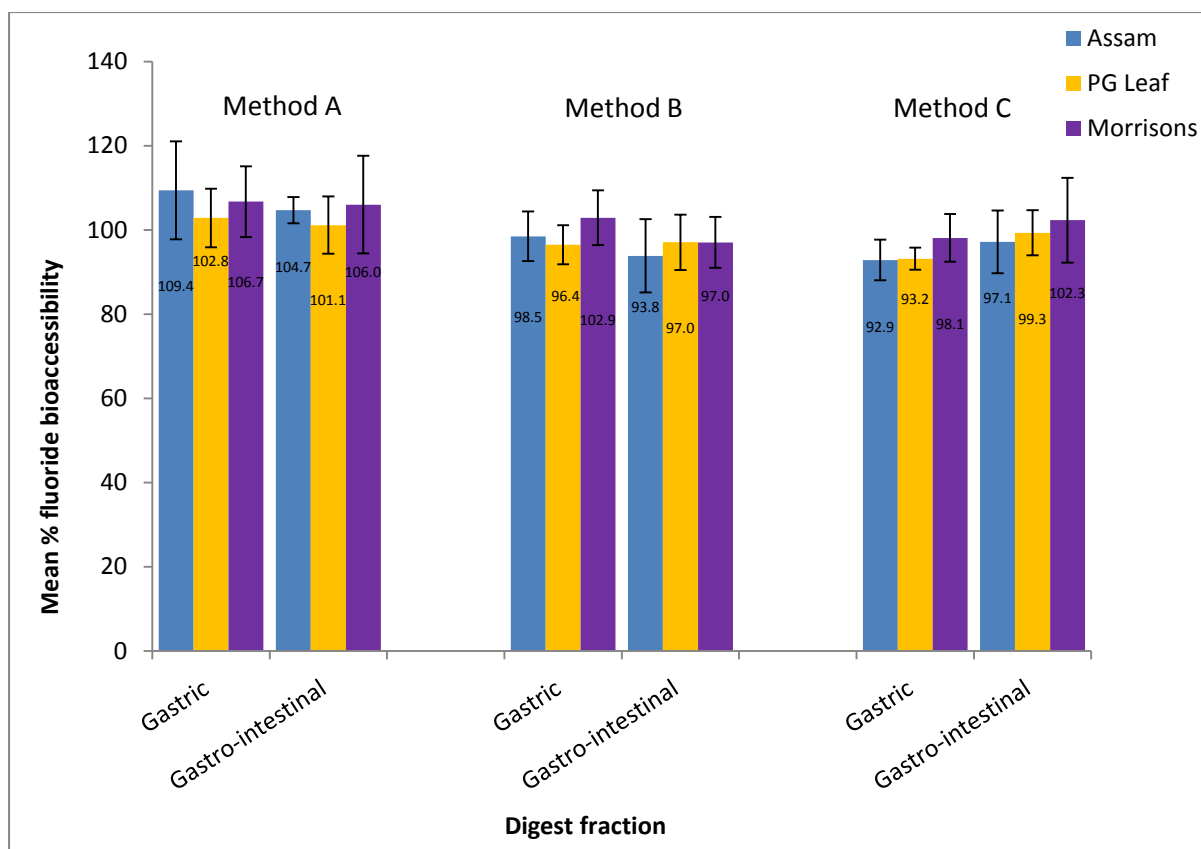


Figure 5.3 Mean percentage fluoride bioaccessibility in the digest fractions for Methods A, B and C (mean  $\pm$  SD, n=4)

### 5.3.2 Bioaccessibility assessment

#### *Bioaccessibility of fluoride in tea infusions and tea leaf precipitate*

Results for the bioaccessibility of fluoride in the three tea products, using the developed Method C, are shown in Table 5.3. Overall mean fluoride concentrations in the fractions for all the tea products ranged from  $1.2 \pm 0.1$  to  $5.9 \pm 0.2$  mg l<sup>-1</sup>.

Fluoride concentration in Twinings Assam bags ranged from  $1.1 \text{ mg l}^{-1}$ , in the initial infusion, to  $1.4 \text{ mg l}^{-1}$  after the gastro-intestinal digestion. PG Tips leaf fluoride concentration ranged from  $4.3 - 5.0 \text{ mg l}^{-1}$ , following gastro-intestinal digestion. Morrisons Value bags 2, having significantly higher fluoride concentrations ( $p < 0.001$ ), ranged from  $5.5 - 6.2 \text{ mg l}^{-1}$  in the fractions. The order of fluoride concentration in the gastric digestion and gastro-intestinal digestions is Morrisons Value 2 bags > PG Tips leaf > Twinings Assam bags.

For the individual tea products ( $n=10$ ) against the digestion fractions, no significant differences ( $p < 0.05$ ) in fluoride concentrations were found in Morrisons Value bags 2 between the infusions or either digest fractions. Significant increases in fluoride concentration were observed between the infusions and gastric fraction and between the gastric and gastrointestinal fractions for PG Tips leaf ( $p < 0.001$ ). For Twinings Assam bags, a significant increase was observed between the infusions and gastrointestinal fraction only ( $p < 0.01$ ).

The tea leaf precipitate was the dried residue of solid tea left from preparing the initial infusion and the results for this undergoing digestion are also shown in Table 5.3. Overall, mean fluoride concentrations in the tea precipitates ranged from  $0.9 \pm 0.1 \text{ mg l}^{-1}$  to  $3.1 \pm 0.1 \text{ mg l}^{-1}$ . Twinings Assam bags had significantly lower fluoride concentrations ( $p < 0.001$ ) compared to the other tea products for both digest fractions. PG Tips leaf and Morrisons Value bags 2 produced similar results in the gastric and gastro-intestinal fraction ( $p > 0.05$ ).

The order of fluoride concentration in the tea leaf precipitates after the gastric digestions is Morrisons Value bags 2  $\geq$  PG Tips leaf > Twinings Assam bags. The order of fluoride concentration in the tea leaf precipitates after the gastro-intestinal digestions is PG Tips leaf > Morrisons Value bags 2 > Twinings Assam bags.

Table 5.3 Mean fluoride concentration [ $F^-$ ]  $\text{mg l}^{-1}$  in the digest fractions for the bioaccessibility assessment (n/a = not applicable) (mean  $\pm$  SD)

Product	Digest fraction	Infusion (n=10)	Tea leaf precipitate (n=5)
Twinings Assam bags	Infusion	1.2 $\pm$ 0.1	n/a
	Gastric	1.2 $\pm$ 0.1	0.9 $\pm$ 0.1
	Gastro-intestinal	1.3 $\pm$ 0.1	1.0 $\pm$ 0.1
PG Tips leaf	Infusion	4.8 $\pm$ 0.2	n/a
	Gastric	4.0 $\pm$ 0.2	2.6 $\pm$ 0.1
	Gastro-intestinal	4.7 $\pm$ 0.2	3.1 $\pm$ 0.1
Morrisons Value bags 2	Infusion	5.9 $\pm$ 0.2	n/a
	Gastric	5.6 $\pm$ 0.3	2.6 $\pm$ 0.2
	Gastro-intestinal	5.8 $\pm$ 0.2	2.9 $\pm$ 0.2

The percentage of fluoride bioaccessibility from each tea product is shown in Figure 5.4 using the mean data from Table 5.3. Similar to when Method C was employed in the method development (Figure 5.3), the amount of fluoride bioaccessibility increased from the gastric fraction into the gastrointestinal fraction for all three tea products. For Twinings Assam bags, over 100% of the fluoride appeared to be available for absorption in the gastric fraction. The order of percentage fluoride bioaccessibility in the tea infusions is Twinings Assam bags > Morrisons Value bags 2 > PG Tips leaf.

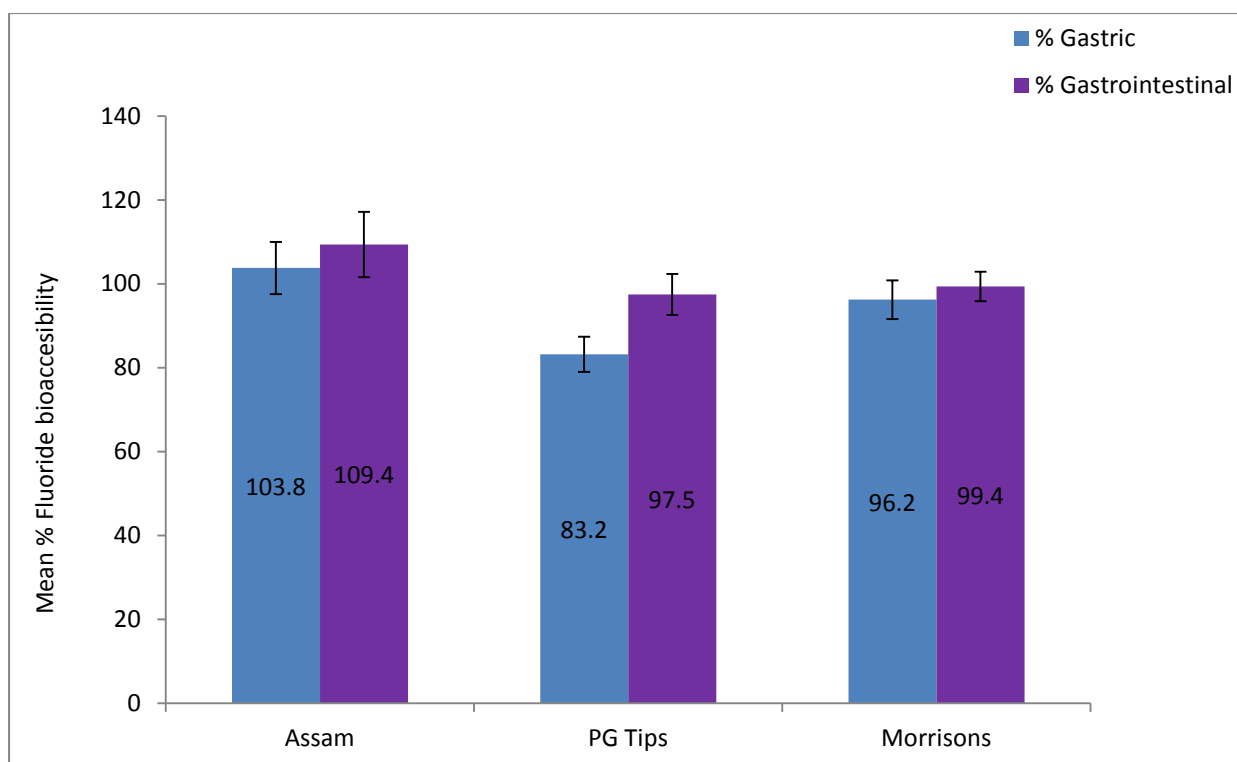


Figure 5.4 Mean percentage fluoride bioaccessibility in the digest fractions (mean  $\pm$  SD, n=10)

#### ***Bioaccessibility of fluoride in tea infusions with the addition of saliva and milk***

Results from the addition of saliva or milk to the digestion process of the tea infusions are given in Table 5.4.

Table 5.4 Mean fluoride concentration  $[F^-]$   $\text{mg l}^{-1}$  in the digest fractions with the addition of saliva and milk (mean  $\pm$  SD)

Product	Digest fraction	Infusions (n=10)	Addition of saliva mean & SD (n=5)	Addition of milk mean & SD (n=5)
Twinings Assam bags	Gastric	1.2 $\pm$ 0.1	1.2 $\pm$ 0.1	1.2 $\pm$ 0.0
	Gastro-intestinal	1.3 $\pm$ 0.0	1.3 $\pm$ 0.0	1.3 $\pm$ 0.1
PG Tips leaf	Gastric	4.0 $\pm$ 0.2	4.4 $\pm$ 0.3	3.5 $\pm$ 0.2
	Gastro-intestinal	4.7 $\pm$ 0.2	4.4 $\pm$ 0.1	4.0 $\pm$ 0.1
Morrisons Value bags 2	Gastric	5.6 $\pm$ 0.3	5.7 $\pm$ 0.2	5.4 $\pm$ 0.2
	Gastro-intestinal	5.8 $\pm$ 0.2	6.0 $\pm$ 0.4	6.5 $\pm$ 0.2

When simulated saliva was added, a significant increase in fluoride concentration ( $p<0.05$ ) was observed for PG Tips leaf in the gastric fraction, but this decreased in the gastro-intestinal fraction ( $p<0.05$ ) when compared to the results without the addition of saliva (Table 5.4). Comparing the fluoride concentration in the gastric fraction with the gastrointestinal fraction, a significant increase in fluoride concentration was found in Twinings Assam bags ( $p<0.05$ ) but not in PG Tips leaf or Morrisons Value bags 2.

The percentage fluoride bioaccessibility with the addition of saliva is shown in Figure 5.5. An increase in percentage fluoride bioaccessibility from the gastric fraction to the gastrointestinal fraction is shown for all the three tea products. The order of percentage fluoride bioaccessibility with the addition of saliva in the gastric fraction is Morrisons Value bags 2 > Twinings Assam bags > PG Tips leaf. In the gastrointestinal fraction, the order followed the same as without saliva, Assam bags > Morrisons Value bags 2 > PG Tips leaf.

The addition of milk, Figure 5.6, decreased the fluoride concentration in the gastric and gastrointestinal fraction significantly compared to without milk in the infusions ( $p<0.01$ ) for PG Tips leaf and in the gastric fraction only for Morrisons Value bags 2 ( $p<0.001$ ). Fluoride concentration significantly increased from the gastric to gastrointestinal fraction ( $p<0.05$ ) for all tea products with milk.

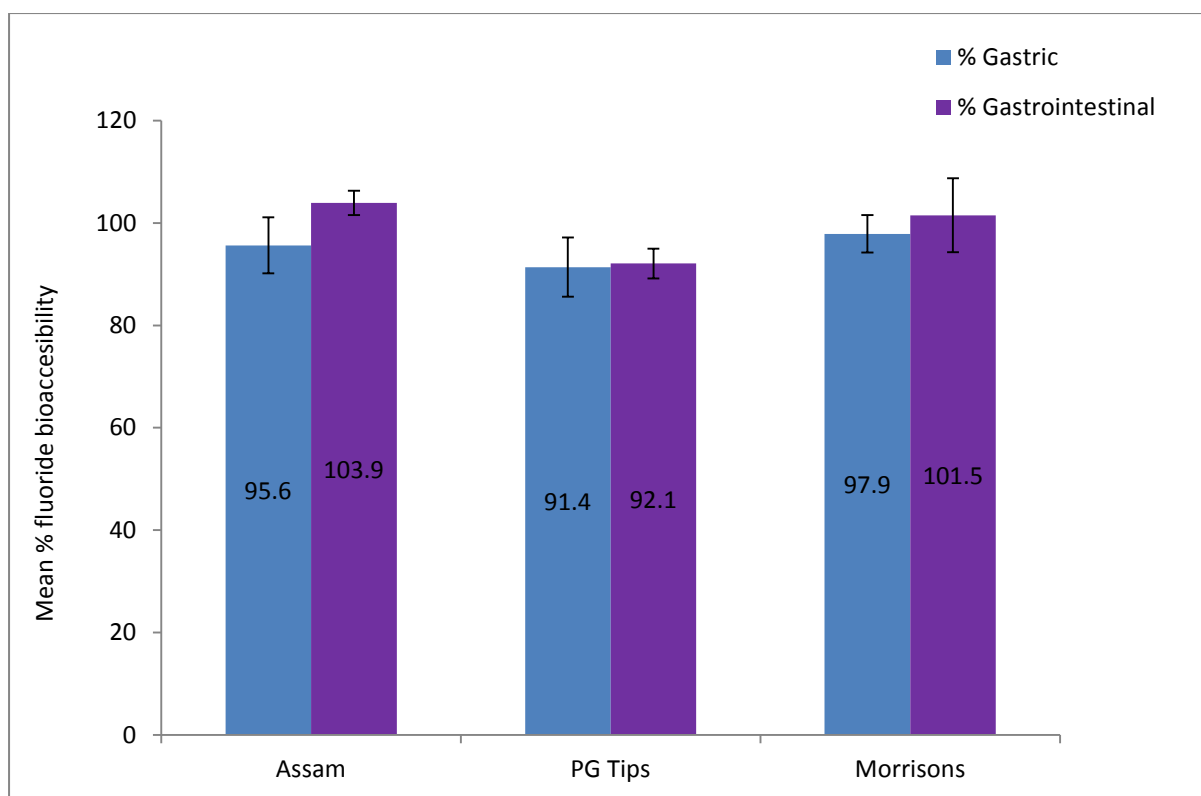


Figure 5.5 Mean percentage fluoride bioaccessibility with the addition of saliva to the tea infusions (mean  $\pm$  SD, n=5)

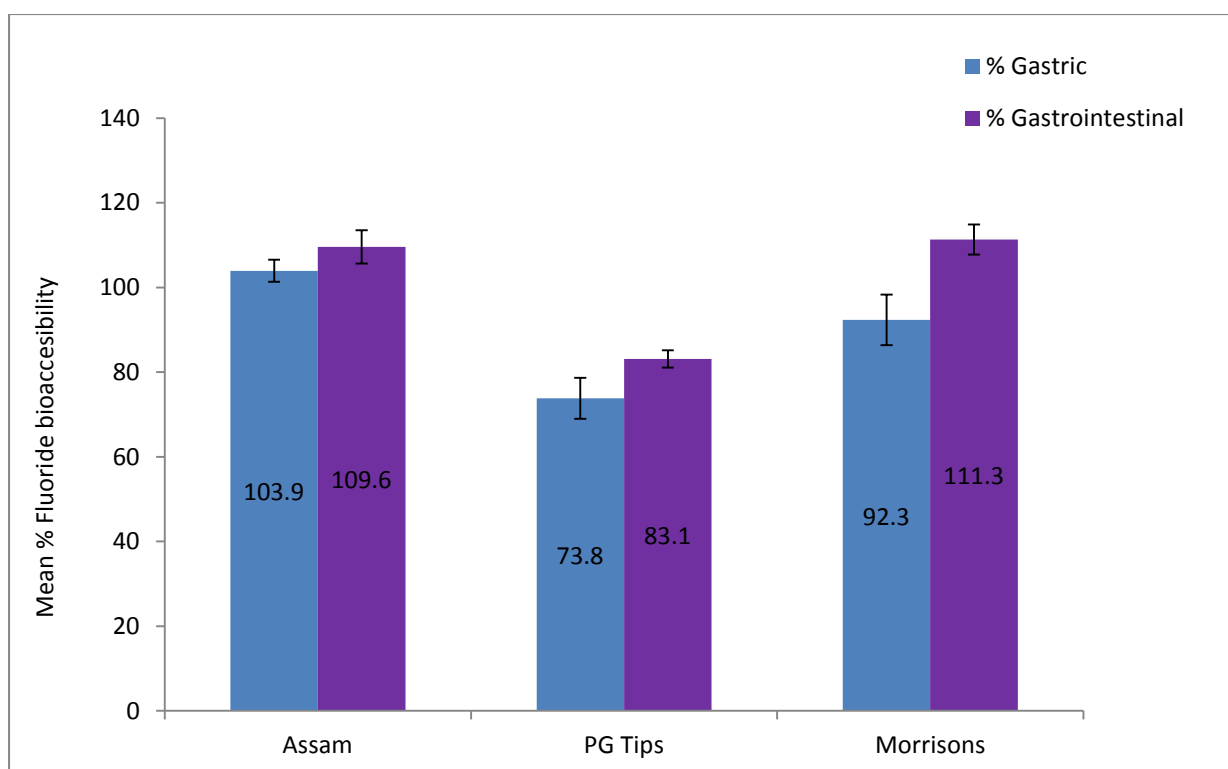


Figure 5.6 Mean percentage fluoride bioaccessibility with the addition of milk to the tea infusions (mean  $\pm$  SD, n=5)



### ***Bioaccessibility of fluoride in the tea leaf product***

Mean bioaccessibility fluoride results for the dried tea products are given in Table 5.5, showing an overall range of  $2.9 \pm 0.1$  to  $13.0 \pm 0.6 \text{ mg l}^{-1}$ . The order of fluoride concentration in the tea leaf products for both the gastric and gastro-intestinal digestions is Morrisons Value 2 bags > PG Tips leaf > Twinings Assam bags. Significant differences in available fluoride are observed between the gastric and gastro-intestinal fractions for all tea products ( $p > 0.001$ ).

Table 5.5 Mean fluoride concentration [ $\text{F}^-$ ]  $\text{mg l}^{-1}$  in the digest fractions from the tea product (mean  $\pm$  SD)

Product	Digest fraction	Tea leaf product (n=5)
Twinings Assam bags	Gastric	$2.9 \pm 0.1$
	Gastro-intestinal	$4.1 \pm 0.2$
PG Tips leaf	Gastric	$7.0 \pm 0.2$
	Gastro-intestinal	$11.2 \pm 0.5$
Morrisons Value bags 2	Gastric	$8.0 \pm 0.3$
	Gastro-intestinal	$13.0 \pm 0.6$

### **5.3.3 Quality control**

Results for the CRM recovery, instrument blank and percentage standard recovery undergoing the digestions are shown in Table 5.6. All methods gave a fluoride concentration recovery of over 85% for CRM NCSZC76304, with Method A showing

the highest recovery of  $88.7 \pm 4.3$  %. However the CRM was an indication of the instrumental performance rather than the individual methods. The blanks for all methods detected less than  $0.05 \text{ mg l}^{-1}$  of fluoride, indicating minimal fluoride contamination.

Percentage standard recovery of the  $5 \text{ mg l}^{-1}$  standard was the lowest for Method B and the standard deviation was the highest. Method C gave the highest percentage recovery of the fluoride standard and the lowest standard deviations.

Table 5.6 Quality control results for Methods A, B and C

	n	% recovery of CRM NCSZC76304 ( $1 \text{ mg l}^{-1}$ ) (mean & SD)	Blank [F <sup>-</sup> ] concentration	% Standard recovery ( $5 \text{ mg l}^{-1}$ ) gastric digestion (mean & SD)	% Standard recovery ( $5 \text{ mg l}^{-1}$ ) gastro-intestinal digestion (mean & SD)
Method A	9	$88.7 \pm 4.3$ %	$<0.05 \text{ mg l}^{-1}$	$86.0 \pm 8.7$ %	$94.7 \pm 4.2$ %
Method B	6	$86 \pm 4.4$ %	$<0.05 \text{ mg l}^{-1}$	$86.0 \pm 8.5$ %	$84.0 \pm 16.9$ %
Method C	5	$85.8 \pm 3.6$ %	$<0.05 \text{ mg l}^{-1}$	$89.5 \pm 6.8$ %	$93.5 \pm 1.9$ %

The quality control results obtained for the developed bioaccessibility assessment are shown in Table 5.7. For the instrumental performance, precision and accuracy was achieved. The quality control measures for the method validation included running a sample blank measuring  $<0.05 \text{ mg l}^{-1}$  of fluoride. The percentage standard recovery of a  $5 \text{ mg l}^{-1}$  fluoride standard after the digestion fractions was over 92.7 % indicating accurate fluoride recovery using the digestion methods.

Table 5.7 Quality control results for bioaccessibility assessment (n/a = not applicable)

Sample	n	[F <sup>-</sup> ] concentration (mean ± SD)	% Mean recovery
CRM NCSZC76304 (1 mg l <sup>-1</sup> )	20	0.8 ± 0.1 mg l <sup>-1</sup>	83.8 ± 5.9 %
Instrument blank	20	< 0.05 mg l <sup>-1</sup>	n/a
Instrument standard recovery (6 mg l <sup>-1</sup> )	17	5.8 ± 0.2 mg l <sup>-1</sup>	95.7 ± 2.7 %
Method standard recovery (5 mg l <sup>-1</sup> ) gastric digestion	17	4.6 ± 0.2 mg l <sup>-1</sup>	92.7 ± 4.4 %
Method standard recovery (5 mg l <sup>-1</sup> ) gastro-intestinal digestion	17	4.7 ± 0.2 mg l <sup>-1</sup>	94.2 ± 3.9 %
Sample blank	17	< 0.05 mg l <sup>-1</sup>	n/a

## 5.4 Discussion

### *Method development*

Wide variations of *in vitro* experimental models to evaluate bioaccessibility, depending on the field of scientific research or the matrix of interest, continue to be published in literature (Powell *et al.* 1998; Bermejo *et al.* 2002; Dean 2006; Intawongse and Dean 2008; Fernandez-Garcia *et al.* 2009; Hur *et al.* 2011; Kabak and Ozbey 2012; Leufroy *et al.* 2012; Koch *et al.* 2013). The development of a method for this study was therefore, based on the comparison of results from the different methods, specifically Method A (Powell *et al.* 1998), Method B (Intawongse and Dean 2008) and Method C (Bermejo *et al.* 2002).

Results for Method A had the widest range in fluoride concentration across all the tea products and also exhibited 100% fluoride bioaccessibility in all digest fractions. This method by Powell *et al.* (1998) was not strictly followed, as human gastric juice

was substituted with simulated gastric juice as described by Mounicou *et al.* (2002). The percentage recovery of the 5 mg l<sup>-1</sup> standard in the gastric and gastrointestinal digest fractions were 86.0% and 94.7%, respectively, whereas the percentage bioaccessibility of fluoride in the tea products was ~100%, Figure 5.3. This variability could be a function of adaptation in the execution of the methods, operating procedures and possible sample evaporation, as the gastrointestinal incubation time for this method was the longest of all the three methods studied, 24 hours compared to Method B at 4 hours and Method C at 60 minutes.

Method B was based on the bioaccessibility of trace metals in a solid matrix of vegetable matter (Intawongse and Dean 2008). Although this is a well documented method, applying this to a liquid matrix meant that the weight to volume ratios could not be reproduced (Cui and Chen 2010; Gbefe *et al.* 2011; Karadas and Kara 2011; Intawongse *et al.* 2012; Wragg and Cave 2012). Despite this, fluoride concentrations in the gastric fractions were similar to Method A and C. The percentage standard recovery in the quality control gave the highest standard deviations, Table 5.6. This method used bile salt in the gastro-intestinal conditions, whereas the other methods did not. Bile salt is a complex salt mixture, which can affect the pH of the small intestine (Danielsson and Sjovali 1985). This may have led to variability in this study. Additionally, the percentage fluoride bioaccessibility in the tea products bore no trends, Figure 5.3.

Method C was chosen to be the developed method for further study as being the most precise, having the lowest standard deviations for fluoride concentrations in the standards and tea samples. The percentage fluoride bioaccessibility in the tea products followed a similar trend to the percentage standard recovery of the 5 mg l<sup>-1</sup> fluoride standard in the digestion fractions; see Table 5.6 and Figure 5.3. Both standard and tea products exhibited a similar increase in fluoride concentration from the gastric to the gastrointestinal fraction. In addition, the percentage standard

recovery ( $5 \text{ mg l}^{-1}$ ) produced the lowest standard deviation compared with the other methods tested, indicating precision, Table 5.6. The more reproducible results from this method were probably due to it being a modification of the study on human and infant formula milk by Bermejo *et al.* (2002). This was based on a liquid matrix, comparable with the liquid tea infusions used in this study, despite the analyte of interest being different.

### ***Bioaccessibility assessment***

As mentioned in Chapter 4, Section 4.4.1 b, particle size affected the diffusion rate of fluoride leaching into the infusion, a similar pattern was observed for the tea products undergoing digestions (Gulati *et al.* 1993; Ma *et al.* 2012). A visual estimation of particle size was in the order of PG Tips leaf > Twinings Assam bags > Morrisons Value bags 2 (appendix 1). With Morrisons Value bags 2 having the smallest particle size, no significant differences were observed between the infusion and digestion fractions, but significant increases were observed for the larger particle sized PG Tips leaf and Twinings Assam bags ( $p < 0.01$ ), Table 5.3. This suggested that fluoride release from the infusion to the other digest fractions was different depending upon the teas particle size.

A significantly lower concentration of fluoride ( $p < 0.01$ ) was left in the tea leaf precipitate, compared to what was available in the infusions, Table 5.3. Although this was removed in the developed method using a Whatman No.1 filter paper, it should be considered that if a consumer prepares a tea beverage from a tea bag, some of these finer particles may be present in their infusion. The mesh size of a tea bag, approximately  $200 \mu\text{m}$  (FATA 2013), is much coarser than a laboratory graded filter paper,  $11 \mu\text{m}$  for Whatman No.1 (Whatman 2013), therefore tea leaf residue can be a further source of fluoride in the diet.

Percentage fluoride bioaccessibility increases later into the digestion process, in the small intestine, for all of the tea products, despite over 100% bioaccessibility for Twinings Assam leaf, this increase is still observed, Figure 5.4. Although the original study published by Bermejo *et al.* (2003) was based on the bioaccessibility of iron and zinc. An increase in concentration for both analytes was reported from the gastric digestion to the gastrointestinal fractions (Bermejo *et al.* 2003).

There is no published data, concerning *in vitro* experiments of fluoride bioaccessibility from tea. However, according to Mahvi *et al.* (2006), around 95% of fluoride released into a tea infusion is estimated to be absorbed by the body, which is in agreement with the percentages of fluoride bioaccessibility observed in this study, Figure 5.4. It should be noted that the experiments described by Mahvi *et al.* (2006) do not explain how this percentage was obtained. Trautner and Sierbert (1986) conducted an *in vivo* study of fluoride bioavailability in humans from a range of foods. Tea was among the foods tested and results showed a rapid absorption of fluoride from tea in the blood plasma, reaching a maximum after approximately 30 minutes. Trautner and Siebert (1986) also report a relative percentage availability of fluoride of 89%.

The effect of the addition of saliva to the digestions did not make a statistical difference ( $p>0.05$ ) for two out of the three tea products, Table 5.4. This suggests the effect of saliva did not alter the fluoride available for absorption for Morrisons Value bags 2 or Twinings Assam bags; however the inclusion of saliva represented a more typical human digestion system (Hur *et al.* 2011). Fluoride analysis of the tea infusion represented in the oral cavity fraction was not quantified due to the limited time a beverage would be present in a real human system. Simpson *et al.* (2001) analysed the oral cavity fraction from the effect of tea rinsing for one minute using human subjects. Interestingly 34% of fluoride from black tea rinsing (with 8 ml of black tea for 60 seconds) will bind to enamel particles on the tooth surface (Simpson *et al.*

2001), but this represented using the tea beverage as a mouth wash, which was not representative of consumption.

Simulated saliva, in this study, was prepared with the addition of mucin, which is a main component of mucus (Poinot *et al.* 2009). Mucus can act as a barrier for ions and can affect fluoride transportation in the human intestine. The static *in vitro* set up described may not reflect this effect, as permeability of the intestine was not included (Wikman *et al.* 1993, Rocha *et al.* 2012). However, despite this exclusion the results do indicate further fluoride is available for absorption in the small intestine, despite the enzymatic effect of the saliva initially into the infusion, Table 5.4.

Figure 5.6 shows the percentage fluoride bioaccessibility with the addition of milk, calculated using the infusion data. A reduction in available fluoride is observed, suggesting that the addition of milk interferes with fluoride availability in the stomach, but further fluoride becomes available in the small intestine. Milk contains calcium and this can produce insoluble complexes with fluoride, such as calcium fluoride ( $\text{CaF}_2$ ) in certain foods (Cerklewski 1992, 1997). Insoluble fluoride complexes could have been removed from the gastric fraction when it was centrifuged, hence the decrease in fluoride concentration. The addition of milk and milk products are reported to reduce the availability of fluoride by 30% when in the form of sodium fluoride, in the human fasted state (Trautner and Einberg 1989). Similar observations were reported by Maddaloni *et al.* (1998) for other chemicals in studies with human and animal bioassays.

Although human ingestion of unprepared solid tea product would not usually occur, the three tea products were digested using the developed method to determine how much fluoride could be available. As mentioned in Chapter 3, there are practices of using tea in a salad or as a pickled condiment (Chi and Jackson 2011). Fluoride concentrations in the solid dried tea products are significantly higher than the

available fluoride in the infusions for both digest fractions ( $p < 0.01$ ). With fluoride in the gastric fraction ranging from 2.8 - 8.4 mg l<sup>-1</sup>, depending upon the tea product, the ingestion of dried or fermented tea as a direct food source could lead to detrimental health effects.

A comparison of the sum of fluoride bioaccessibility concentration in the digestion fractions added to the amount in the tea leaf precipitate was compared to the total in the digestion fractions of tea leaf product (Table 5.8). If the digestion of the tea had broken down all the fluoride in the tea samples, the sum of the infusion and precipitate would be equal to the concentrations of fluoride in the tea leaf products. A significant increase in fluoride bioaccessibility was observed in the gastrointestinal digestion for all of the tea leaf products ( $p > 0.01$ ). This suggests that the overall digestion time of the developed method of 2 hours may not be sufficient for the complete breakdown of fluoride complexes within a solid matrix.

Table 5.8 Mean fluoride bioaccessibility concentration in digestion fraction + tea leaf precipitate compared to tea leaf product [F<sup>-</sup>] mg l<sup>-1</sup> (mean ± SD)

Product	Digest fraction	Infusion + precipitate (n=5)	Tea leaf product (n=5)	Difference
Twinings Assam bags	Gastric	2.1 ± 0.1	2.9 ± 0.1	+ 0.8
	Gastro-intestinal	2.3 ± 0.1	4.1 ± 0.2	+ 1.8
PG Tips leaf	Gastric	6.6 ± 0.1	7.0 ± 0.2	+ 0.4
	Gastro-intestinal	7.8 ± 0.1	11.2 ± 0.5	+ 3.4
Morrisons Value bags 2	Gastric	8.2 ± 0.2	8.0 ± 0.3	- 0.2
	Gastro-intestinal	8.7 ± 0.2	13.0 ± 0.6	+ 4.3



Powell *et al.* (1998) Method A, adopted a total digestion time of 25 hours in their study which was carried out on tea infusions also, but used specialised equipment (Centricon microconcentrator tubes with Ultrafilters) which was unavailable for this study. Method B (Intawongse and Dean 2008) used a total digestion time of 5 hours for solid vegetable matrices. Method C may have required a longer gastrointestinal digestion time similar to Intawongse and Dean (2008) to complete the digestion of fluoride. Considerations should be made as Intawongse and Dean (2008) used a solid matrix and therefore, natural digestion periods would usually be longer than the developed method incubation time, which was based on digesting a liquid.

From this study, fluoride in tea is found available for absorption in the simulated model of human stomach conditions, including potential absorption in the oral cavity with 91.4 - >100% bioaccessibility of fluoride in the gastric compartment. Further absorption can occur in the small intestine as increased bioaccessibility of fluoride was shown to occur in this fraction, 92.1 - >100%, depending upon the tea product (Figure 5.6). When comparing this to the literature, studies describing the oral bioavailability of fluoride are documented through *in vivo* studies using rats or humans (Trautner and Siebert 1986; Trautner and Einwag 1989; Rigalli *et al.* 1994; Buzalaf *et al.* 2008; Suarez *et al.* 2008). Many factors can affect the oral bioavailability of fluoride, for example if fluoride is covalently bonded, as sodium monofluorophosphate, absorption into the blood is slower than ionic fluoride, such as sodium fluoride (Trautner and Siebert 1986; Trautner and Einwag 1989; Buzalaf *et al.* 2008). If fluoride is presented as ionic sodium fluoride, a 100% absorption into blood plasma (of rats) is observed and at a rapid rate (Rigalli *et al.* 2004). Trautner and Siebert (1986) compared the absorption of fluoride from tea in human subjects to having a similar rate to sodium fluoride, with peak fluoride plasma concentrations occurring after 30 minutes.

A decrease in pH is reported to affect fluoride absorption, favoured under prevailing acidic conditions (Nopakum and Messer 1990; Messer and Ophaug 1993; He *et al.* 1998; Rocha *et al.* 2012). In a tea infusion the majority of the fluoride is in the ionic form (Horie *et al.* 1992). With the conditions of the stomach being highly acidic, ionic fluoride can be protonated into the weak undissociated acid, hydrofluoric acid which allows the fluoride to pass through the mucus layer by passive diffusion (Whitford and Pashley 1984; Buzalaf and Whitford 2011, Rocha *et al.* 2012). The developed method in this study mimicked the acidic conditions of the stomach at pH 2 and it is possible that hydrofluoric acid did form within the gastric fraction. According to Buzlaf and Whitford (2011), further fluoride can be absorbed in the small intestine; however, the pH is neutral in this compartment, simulated at pH 7 in this study. Therefore, the same mechanism for fluoride absorption in the form of hydrofluoric acid would not occur in the gastrointestinal fraction.

With such a high percentage of fluoride being available from tea from digestion, the metabolism should also be considered. According to *in vivo* studies, where fluoride is rapidly absorbed (20 - 60 minutes peak plasma fluoride concentrations); a rapid decline in plasma fluoride concentration is also observed (Trautner and Siebert 1986; Trautner and Einwag 1989; Buzalaf *et al.* 2008). Buzalaf and Whitford (2011) explain the elimination of fluoride from plasma as being via urine excretion or by being taken into calcified body tissues. Elimination through the urine is observed after a few hours (Buzalef *et al.* 2008; Suarez *et al.* 2008). Approximately 50% of absorbed fluoride will be excreted in the urine or faeces following human ingestion within 24 hours; the remainder is readily absorbed in the oral cavity, stomach and small intestine (Whitford 1996; Cerklewski 1997; Simpson *et al.* 2001; Cao *et al.* 2001a; Inkielewicz and Czarnowski 2008). Almost all fluoride in the body is taken up into the skeletal part of the body (Cerklewski 1997). Fluoride binds to enamel particles on the tooth surface (Simpson *et al.* 2001), due to the high reactivity and the small ionic radius of fluoride allowing it to replace hydroxylapatite ions in the tooth enamel as

the more resistant ion, fluorapatite (Cerklewski 1997). Although the metabolism and oral cavity effects were not simulated in this study, it offers a purpose for further study.

As previously established, fluoride concentration is extremely variable depending upon the tea product and brewing time. The order of percentage fluoride bioaccessibility gave a similar order to fluoride concentrations determined in the tea products and infusions (Chapters 3 and 4), Economy tea > Black tea > Pure tea. However, despite the tea product used, a high percentage of fluoride is available for absorption in the stomach with further absorption in the small intestine (up to 100% of the tea fluoride content from the infusion). If consuming an economy branded tea, a higher concentration of fluoride will be available for absorption by the human system by almost 3 fold when compared to a pure blend, such as Twinings Assam bags.

Although a certified reference material does not exist to validate the method, this study has demonstrated an *in vitro* experiment. This novel approach can form the foundations for further research for *in vitro* extractions of fluoride bioaccessibility from tea infusions and other beverages.

## 5.5 Chapter Summary

- Using a laboratory approach, an *in vitro* method was developed to assess the bioaccessibility of fluoride in tea infusions. This method may also be suitable for a range of beverages or food in liquid matrices.
- Bioaccessibility of fluoride in tea infusions ranges from 91.4 - 100% in the gastric compartment of human digestion, with further fluoride available for

absorption in the small intestine (gastrointestinal fraction), ranging from 92.1 - 100%.

- The bioaccessibility of fluoride is independent on fluoride concentration in the tea product, with Twinings Assam bags, a low fluoride concentration tea product and Morrisons Value bags 2, a high fluoride concentration product, exhibiting a higher percentage fluoride bioaccessibility than a mid range tea, PG Tips leaf.
- Including saliva to the developed method did not have an overall effect on the bioaccessibility of fluoride from the tea infusions, but represented a more realistic digestion model of the human system.
- The fluoride bioaccessibility concentration is dependent upon brand, where Morrisons Value bags 2 can deliver  $6.0 \text{ mg l}^{-1}$  of available fluoride to the human system compared to  $1.3 \text{ mg l}^{-1}$  when consuming pure blended Twinings Assam bags, after gastrointestinal digestion.
- Excess fluoride from tea leaf precipitate or residue left in a tea cup from brewing a beverage could continue to leach fluoride and increase the total fluoride concentration available for absorption.

## **Chapter 6: Fluoride uptake and accumulation from soil by *Camellia sinensis***

## 6.1 Introduction

Plants can be exposed to fluoride in the environment from the soil by either natural occurrence or anthropogenic contamination, or from airborne sources (Kabata-Pendias 2010). Natural fluoride in soil mostly occurs from weathering of minerals in the Earth's crust, but can also be present from deposition of airborne sources (Davison 1983). Anthropogenic contamination occurs from a wide range of sources, such as application of organic and inorganic fertilizers and pesticides, pharmaceutical residues and from mining (Weinstein and Davison 2004). Airborne fluoride can occur from industrial sources such as aluminium smelters, as well as natural sources like volcanic activity, affecting plants in the vicinity, with inhibition of growth and/or morphological damage being the first signs of excessive exposure (Weinstein 1977; Halmer *et al.* 2002; Weinstein and Davison 2004; Koblar *et al.* 2011). Fluoride toxicity affects plants respiratory rate, thus altering normal growth patterns (Miller 1993). Deposits of fluoride in gaseous and particulate forms can be deposited onto external plant surfaces where uptake into the tissues can occur (Davison 1983; Weinstein and Davison 2004). Many environmental factors affect the rate of uptake from airborne sources, such as temperature, wind direction and speed and rain (Israel 1974; Schwela 1979; Weinstein and Davison 2004). Different plants species growing in the same location have been reported to accumulate different concentrations of fluoride from airborne sources (Weinstein and Davison 2004).

The most common natural terrestrial fluoride bearing minerals are calcium fluorophosphate (fluorapatite), calcium fluoride (fluorspar) and sodium hexafluoroaluminate (cryolite) (Cooke *et al.* 1976). Fluoride from the soil can be taken up through plant roots by passive diffusion and transported through the root water system in the cell walls and intercellular spaces, the apoplast (Davison 1983; Takmaz-Nisancioglu and Davison 1988; Geeson *et al.* 1998). This route is suggested,

rather than fluoride passing through the cell membrane, the symplast, as the endodermis may prevent fluoride anions passing through (Takmaz-Nisancioglu and Davison 1988). However, if in the HF form, fluoride can enter the cell membrane and form the  $F^-$  ion once inside the cell, allowing the transportation through the symplast (Kronberger 1988). However, the actual pathway is not completely understood (Zhang *et al.* 2013).

Many factors affect the rate of fluoride uptake in plants, especially soil pH. In acidic soils of less than pH 5, fluoride is released into solution and available to complex with aluminium which may aid absorption into the roots (Larsen and Widdowson 1971; Wenzel and Blum 1992; Stevens *et al.* 2000). Takmaz-Nisancioglu and Davison (1988) found an increase in plant uptake of fluoride in bean plants (*Phaseolus vulgaris* L.) when fluoride was in the form of aluminium-fluoride compared to sodium fluoride additions, similar results were reported by Stevens *et al.* (1997). The suggested mechanism, for this differential uptake, is that the organic matter is released when the aluminium-fluoride complexes are formed, allowing mobility (Davison 1983). In neutral and alkaline soils of pH 6.5 and above, fluoride is strongly adsorbed to the soil and is not as available for uptake by plants due to calcium-fluoride interactions (Wenzel and Blum 1992). Ruan *et al.* (2004) found by adding calcium oxide to soils, the availability of fluoride to tea plants was reduced, as the soil pH was raised.

*C. sinensis* L. is a known accumulator of aluminium and fluoride (Shu *et al.* 2003). Fluoride ( $F^-$ ) will form stable complexes with aluminium ( $Al^{3+}$ ) if free ions of both are available from the soil (Stevens *et al.* 1998). These will accumulate in the leaves despite aluminium and fluoride being non-essential element to plants (Kabata-Pendias 2010). *C. sinensis* can contain an abundance of fluoride and aluminium, especially if exposed to additional airborne sources; whereas other species of plants are phytotoxic if exposed to fluoride (Xie *et al.* 2001). Complexes in order of the rate of uptake by plants in general exist as:  $HF > AlF_2^{2+} > AlF_2^+ > AlF_3 = AlF^- = F^-$  (Weinstein

and Davison 2004). In the form of HF, permeability into the roots is 6 times higher than the anionic form,  $F^-$  (Gutknecht and Walter 1981). In alkaline soil, the anion  $F^-$  would exist and fluoride would be more likely be bound to soil as a complex, however, *C. sinensis* thrives on acidic soils, where fluoride could exist as HF allowing permeability and uptake by the roots (Stevens *et al.* 1997). Fluoride uptake through the roots of *C. sinensis*, is suggested to follow an active process requiring energy, dissimilar to passive transportation by other plant species (Zhang *et al.* 2013). This may account for *C. sinensis* characteristic accumulation of fluoride.

Transfer factors are used to determine elemental distribution within a plant system (Liang *et al.* 2013). They are useful indicators in environmental assessment of soil-to-plant or root-plant transfers (Kabata-Pendias 2004). The majority of studies have used heavy metals, to assess plant uptake from contaminated soil (DeForest *et al.* 2007; Baltrenaite *et al.* 2012; Liang *et al.* 2013); however, Saikat (2004) used transfer factors to determine the uptake of iodide and fluoride by dock (*Rumex sp.*).

Experiments directed on fluoride uptake by *C. sinensis* are limited, mostly carried out on tea plantations or using hydroponic nutrient systems (Fung *et al.* 2003; Shu *et al.* 2003; Ruan *et al.* 2004; Xie *et al.* 2007; Li and Ni 2009). *C. sinensis* is a slow growing shrub and therefore, experiments involving young plants are difficult to carry out on a short term basis. This is an important area of research that should receive increased attention, especially with the elevated concentrations and bioaccessibility of fluoride found in this study, (Chapters 3, 4 and 5). This Chapter reports plant uptake of fluoride and distribution to the parts of the leaf used in the manufacture of the beverage.



The aim of this chapter was to assess the uptake of fluoride by tea plants (*C. sinensis*) and its influence on the plants growth. Towards this aim the following objectives were addressed:

- To administer different doses of fluoride, in the form of sodium fluoride (NaF) to the soil of *C. sinensis* and observe any visual effects over the period including growth pattern.
- To assess the distribution of fluoride in the tea plants root and leaves over time.
- To assess the uptake of fluoride using a different compound of fluoride.
- To determine the influence of fluoride on the growth of tea plants.

## **6.2 Materials and Methods**

### **6.2.1 Materials**

Thirty five *C. sinensis* rooted cutting plants, approximately 18 months old and 20 kg of peat based compost were sourced from Tregothnan Tea Estate, Cornwall, UK. The reagents used were from Sigma-Aldrich. For the analysis of fluoride, reagents were as described previously for IC, Section 2.2.3.2.

## 6.2.2 Methods

### 6.2.2.1 Fluoride dosing

All plants were re-potted into 1 L pots, removing the original compost and replacing with a peat based compost (supplied by Tregothnan Tea Estate and known as 'Camellia special mix'), whilst keeping the root bulb intact. A grid was marked out on a potting table in a glasshouse, where each square measured 20 cm<sup>2</sup>. Each pot was placed on a plastic saucer in the centre of the square and the plants were arranged in a random pattern, Figure 6.1 (Keith 1996). When all the plants were in position on the grid, 100 ml of de-ionised water was added to each pot. The glasshouse temperature was recorded as  $16 \pm 10$  °C and the experiment was carried out over a period of 12 weeks, during spring and summer months. The plant positions on the grid were changed randomly on a weekly basis.

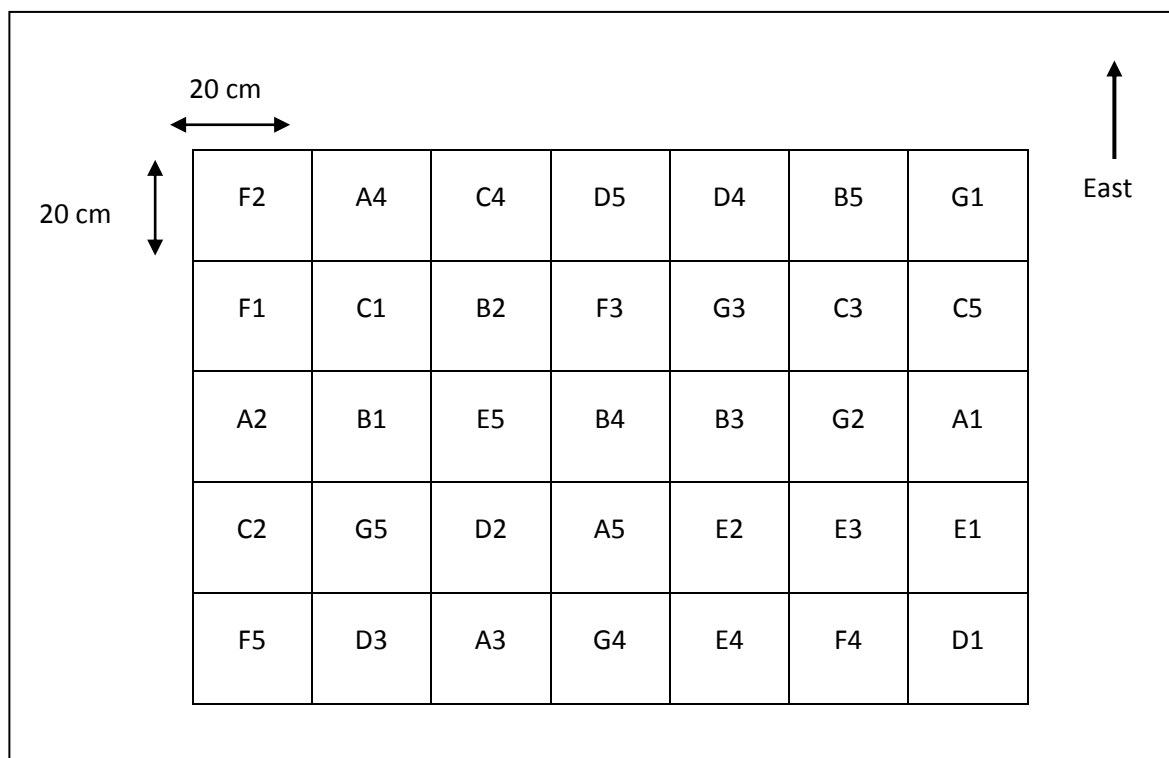


Figure 6.1 Random grid pattern, with labelled pot positions at start of experiment

The codes shown in the grid, Figure 6.1, would represent different fluoride doses to occur after a two week interval, described in '*Dosing*', with 5 sample replicates in each group (1 to 5). A, B, C, E, F and G relate to doses of 50, 100, 150, 200, 0 and 0 mg of fluoride from NaF, respectively. Group D, noted as 150 mg\* of fluoride, as NH<sub>4</sub>F, is highlighted and asterisked throughout as being a different compound of fluoride administered. NaF was chosen as the main compound for dosing, being readily soluble in water, whereas NH<sub>4</sub>F contains nitrogen which is a plant nutrient and this factor could distort the results.

### ***Sampling***

After 2 weeks, the 5 replicates of group G were removed from the grid and harvested to form a blank control sample (Keith 1996). The remaining samples were randomly re-arranged, so no gaps appeared in the grid by omitting the use of the right hand column from the grid. Samples of soil, roots, mature leaves, buds and stem were collected as described below:

Soil was collected by forming a composite from the entire contents of the 5 pots of soil (G1 to G5) and three sub-samples were taken using the coning and quartering technique (McNaught and Wilkinson 1997).

The collected roots consisted of the system of growth from the soil below the stem. These were cut from the stem and composited as one sample, after rinsing with de-ionised water.

Buds consisted of the top bud and the 2<sup>nd</sup> and 3<sup>rd</sup> leaf of each flush. These were trimmed from each plant and composited together as one sample.

The mature leaves consisted of 4<sup>th</sup> leaves and all remaining lower leaves on the plant. These were trimmed, after the removal of 'buds' and composited.

The stem was the woody part of the plant that was left after all the other samples were taken.

The root, bud and leaf composited samples were rinsed with de-ionised water and air dried on plastic trays in the glasshouse for 5 days, prior to being milled and sieved through a mesh size of <125µm and pre-dried at 60°C for 16 hours in Kraft bags (Fung *et al.* 2003). The soils were allowed to air dry, at room temperature, for 7 days before sieving through a 200 µm mesh and transferring into Kraft bags (Fung *et al.* 2003). All samples in Kraft bags were stored in desiccators with silica gel, at room temperature, away from direct sunlight.

### ***Dosing***

The fluoride doses were added to the plants, as described in Table 6.1, to the appropriate plants at Week 2 and Week 6 of the experimental growth period. All plants were watered with the same volume of de-ionised water 3 times a week for the duration of the experiment. This volume varied between 50 and 100 ml with the amount depending upon keeping the soil moist but avoiding over or under watering. After 3 months, the plants were harvested and separated into samples of soil, roots, mature leaves, buds and stems, and stored in Kraft bags as described previously.

Table 6.1 Fluoride doses added to the plants

Plant group	[F <sup>-</sup> ] Dosage	Harvest date
G	0	2 weeks
F	0	3 months
A	50 mg [F <sup>-</sup> ] from NaF	3 months
B	100 mg [F <sup>-</sup> ] from NaF	3 months
C	150 mg [F <sup>-</sup> ] from NaF	3 months
E	200 mg [F <sup>-</sup> ] from NaF	3 months
D	150 mg [F <sup>-</sup> ] from NH <sub>4</sub> F	3 months

#### 6.2.2.2 Plant observations and measurements

Eight morphological features of the tea plant were assessed, based on descriptors for tea from the International Plant Genetic Resources Institute (IPGRI) guidelines (IPGRI 1997). These included the following, measured initially and every 2 weeks after, unless stated otherwise:

- Plant height
- Internodal spacing (first and second)
- Leaf shape (observed initially and after 12 weeks)
- Upper leaf (observed initially and after 12 weeks)
- Leaf margin (observed initially and after 12 weeks)
- Length of mature leaf (every 4 weeks)
- Petiole
- Flushes, note if any appear

Visual observations were noted and photographs were taken throughout the experimental period. Once the plants were harvested, the root systems were observed and measurements of root length were made.

#### **6.2.2.3 Plant analysis**

Total fluoride: In nickel crucibles, 0.5 g of plant material, i.e., root, bud or mature leaf, was accurately weighed to 4 decimal places. The procedure for analysing total fluoride in plant material was followed as described in Section 2.2.2.2 (Sparks *et al.* 1996; Metrohm 2008).

#### **6.2.2.4 Soil analysis**

pH: In 50 ml Schott bottles,  $2.5 \text{ g} \pm 0.01 \text{ g}$  of 200  $\mu\text{m}$  soil, was accurately weighed and 5 ml of de-ionised water added to equal a 1:2 soil-water ratio (Fung *et al.* 2003). The bottles were capped and shaken for an hour at 70 rpm. Once the contents were settled, pH measurements were taken using a Metrohm combined pH electrode.

Water soluble fluoride: In 50 ml Schott bottles, 0.5 g of soil was accurately weighed to 4 decimal places and 25 ml of de-ionised water added (Saikat 2004). The bottles were capped and placed on a mechanical shaker set at 70 rpm for one hour. Fluoride concentration was determined using ion chromatography as described in Section 2.2.3.2 (Metrohm 2008), after filtering the samples through 0.45  $\mu\text{m}$  cellulose nitrate syringe filters.

Total fluoride: In nickel crucibles, 0.5 g of soil was accurately weighed to 4 decimal places and the procedure for determining total fluoride concentration in soils followed as described in Chapter 2, Section 2.2.3.2 followed (Sparks *et al.* 1996; Metrohm 2008).

### 6.2.3 Quality control

All glassware was acid soaked in 10 % (w/v) nitric acid for 16 hours, followed by triple rinsing with de-ionised water prior to use. The plants were watered with de-ionised water throughout the experiment to prevent possible fluoride additions from using standard tap water. Samples labelled G, were used as background controls and the samples labelled F were used as experimental blanks, Table 6.1.

Certified reference materials, soil ZC73006 (fluoride value  $652 \pm 48 \text{ mgkg}^{-1}$ ) from NCS Testing Technology Company Ltd and tea GBW10016 (fluoride value  $57 \pm 15 \text{ mgkg}^{-1}$ ) from LGC Ltd, were used to validate measured fluoride results in soils and plant materials.

The Metrohm pH meter was calibrated using standard pH 4.01 and pH 7.00 buffer solutions, every 10 measurements and the IC was calibrated using standard fluoride solutions ranging from 5 to 25  $\text{mg l}^{-1}$ . A sample blank was extracted with every 10 samples and analysed for fluoride within the batch. All composite samples of soil, roots, buds and mature leaves were analysed in triplicate.

### 6.2.4 Data analysis

Fluoride concentration was converted to  $\text{mgkg}^{-1}$  against the IC calibration curve ( $\text{mg l}^{-1}$ ) using the following calculation:

$$\text{Fluoride (mgkg}^{-1}\text{)} = \frac{\text{IC reading [F}^{-}\text{] mg l}^{-1} \times \text{dilution volume (ml)}}{\text{weight (g)}}$$

Transfer factors were calculated using:

$$\text{Soil transfer factors} = \frac{\text{Total } [\text{F}^-] \text{ mgkg}^{-1} \text{ in part of plant (roots, buds or mature leaves)}}{\text{Total } [\text{F}^-] \text{ mgkg}^{-1} \text{ in soil}}$$

$$\text{Root transfer factors} = \frac{\text{Total } [\text{F}^-] \text{ mgkg}^{-1} \text{ in part of plant (buds or mature leaves)}}{\text{Total } [\text{F}^-] \text{ mgkg}^{-1} \text{ in roots}}$$

Data followed a normal distribution (Section 2.2.5) and comparisons were made using IBM SPSS and Microsoft Excel to calculate descriptive statistics, ANOVA and Students t-tests.

## 6.3 Results

### 6.3.1 Plant observations

#### *Plant height*

Figure 6.2 shows the mean cumulative height growth (n=5) for each group. Increased growth is observed in all plant groups dosed with NaF, ranging from 5.4 - 22.1 cm at start of week 1 to 6.6 - 24.4 cm by week 12. A significant increase in plant height was observed for the control group F, plants during the study ( $p < 0.001$ ), compared to the other dosed plant groups, Figure 6.2. The coefficient of determination ( $R^2$ ) for the cumulative growth pattern for the NaF doses is shown in Table 6.2. The coefficient ( $R^2$ ) order for plant height with time for the NaF doses is 0 mg > 50 mg > 150 mg > 100 mg > 200 mg.



For plants dosed with  $\text{NH}_4\text{F}$ , plant height ranged from 9.5 - 15.3 cm at the start of week 1, increasing to 10.1 - 16.7 cm at week 12, with the coefficient ( $R^2$ ) showing a positive relationship with increased growth of 0.856, with time, Figure 6.2, Table 6.2.

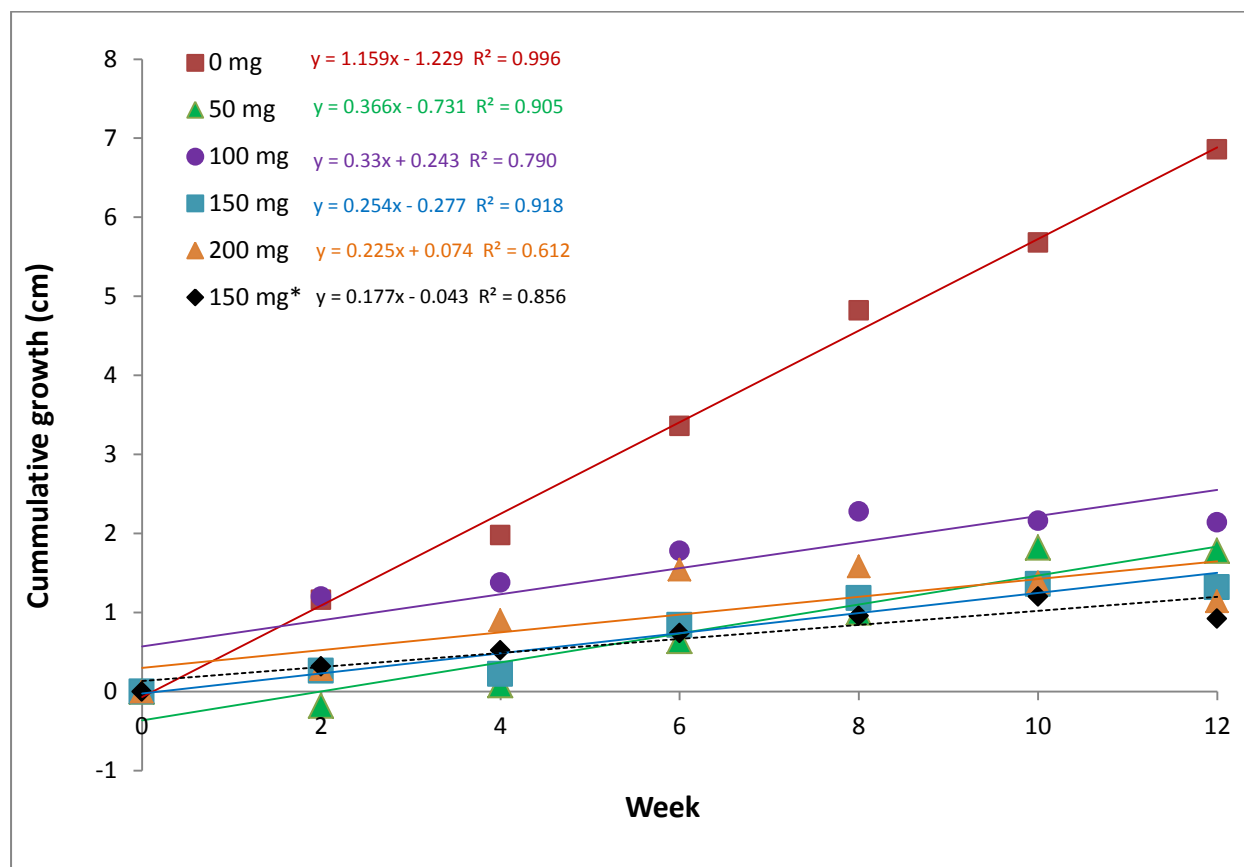


Figure 6.2 Mean cumulative height growth with different fluoride doses, from NaF and  $\text{NH}_4\text{F}$  (n=5)

### **Mature leaf length**

Cumulative mature leaf growth is shown in Figure 6.3 and the  $R^2$  values for the different concentrations from the graph are given in Table 6.2. Mature leaves for the NaF dosed plants showed a significant increase in length as time progressed ( $p < 0.005$ ). The only exception observed was for the highest fluoride dosed group, 200 mg, where no significant difference in length was observed between weeks 8 and 12 ( $p > 0.05$ ), Figure 6.3. This affected the coefficient of determination in mature

leaf growth over time for dose 200 mg, at  $R^2 = 0.850$ , compared to the other doses where a more positive relationship was obtained,  $R^2 \geq 0.947$ , Table 6.2. The order of  $R^2$  for the mature leaf growth over increasing time is 50 mg > 150 mg > 0 mg > 100 mg > 200 mg.

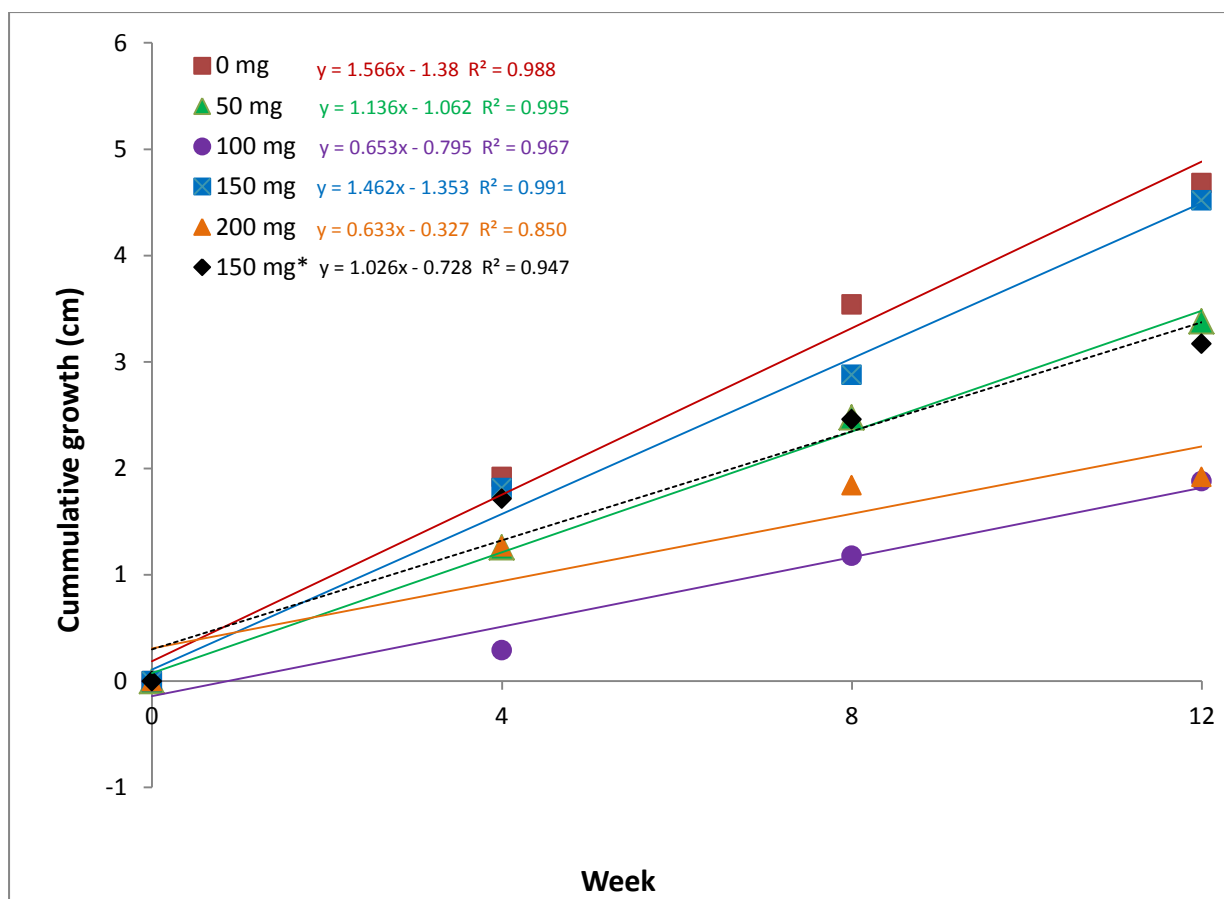


Figure 6.3 Mean cumulative mature leaf growth with different fluoride doses, from NaF and \*NH<sub>4</sub>F (n=5)

The mature leaves of the plants dosed with NH<sub>4</sub>F showed statistical significant increases in growth ( $p < 0.05$ ) and a positive coefficient of  $R^2 = 0.947$  of increasing leaf growth with increasing time, Table 6.2.

Table 6.2 Coefficient of determination for cumulative plant height and mature leaf increase versus time (week) for different fluoride doses (n=5). \*Dosed with  $\text{NH}_4\text{F}$

Group, Dose [ $\text{F}^-$ ]	$R^2$ cumulative plant height growth v week	$R^2$ cumulative mature leaf length v week
F/0 mg	0.996	0.988
A/50 mg	0.905	0.995
B/100 mg	0.790	0.967
C/150 mg	0.918	0.991
E/200 mg	0.612	0.850
D/150 mg*	0.856	0.947

It should also be noted that added fluoride did not affect leaf shape. Leaf shape was described as being mostly lanceolate, with the minority of plants exhibiting elliptic or oblong shapes. The upper leaf was observed as smooth for all plants and the leaf margins were serrulated.

### **Root length**

After harvesting, the structure of the root was observed and measured as length, width and length of primary root, Table 6.3. Root length for the NaF dosed plants ranged from 13.9 to 53.3 cm, width from 6.4 to 15.3 cm and the greatest range was the primary root lengths, from 1.9 to 53.3 cm. The order of increasing root length is 200 mg > 0 mg > 150 mg > 50 mg > 100mg.

Root length ranged from 23.8 to 32.7 cm for the  $\text{NH}_4\text{F}$  dosed plants, appearing to be mid range in length compared to the NaF dosed roots.

Table 6.3 Harvested root measurements (cm), (mean  $\pm$  SD, n=5) \*Dosed with NH<sub>4</sub>F

Group/Dose [F <sup>-</sup> ]	Length	Width	Primary root
F/0 mg	31.4 $\pm$ 12.9	13.08 $\pm$ 1.7	27.0 $\pm$ 17.4
A/50 mg	19.5 $\pm$ 4.3	9.3 $\pm$ 0.8	9.8 $\pm$ 5.7
B/100 mg	18.7 $\pm$ 3.8	8.8 $\pm$ 1.8	9.8 $\pm$ 7.6
C/150 mg	25.4 $\pm$ 6.1	10.5 $\pm$ 1.8	24.1 $\pm$ 8.6
E/200 mg	32.3 $\pm$ 13.1	11.3 $\pm$ 2.7	26.9 $\pm$ 17.1
D/150 mg*	27.7 $\pm$ 4.3	10.0 $\pm$ 2.0	14.8 $\pm$ 14.9

### 6.3.2 Soil pH

Soil pH was measured after harvesting and the mean results are given in Table 6.4. On the pH scale, all soils were classified as acidic ranging from pH 4.44 to 5.15. Statistical differences in pH were observed between all of the soil samples ( $p < 0.03$ ), although for the pH scale, the acidic differences observed in these soils were minimal. The order of the most acidic soil for the different NaF doses was 50 mg > 0 mg > 100 mg > 200 mg > 150 mg.

Soil pH for the NH<sub>4</sub>F dose, was statistically different from the 150 mg NaF dosed soil ( $p < 0.05$ ), but still classified as an acidic soil.

Table 6.4 Soil pH at different fluoride doses (n=5) \* Dosed with NH<sub>4</sub>F

Dose [F <sup>-</sup> ]	Plant reference	Mean pH $\pm$ SD
0 mg	G	4.67 $\pm$ 0.04
0 mg	F	4.50 $\pm$ 0.02
50 mg	A	4.45 $\pm$ 0.02
100 mg	B	4.66 $\pm$ 0.01
150 mg	C	5.08 $\pm$ 0.07
200 mg	E	4.78 $\pm$ 0.01
150 mg*	D	4.86 $\pm$ 0.02

After the initial re-potting, the plants were photographed in their groups of five replicates to represent the visual observation at the start of the experiment. Two weeks later, the plants were becoming established in the compost, as all plants exhibited slight growth with leaves appearing green and healthy. After the measurements were taken, the initial control group G, plants were removed from the experiment and harvested as described in Section 6.2.2.1.

By week 3 of the experimental period, all the plants that had undergone NaF dosing showed evidence of leaf browning to the tips of certain leaves (Plate 6.1) but the control group F, did not show this effect. At week 4, further browning of leaves was observed on all the plants that underwent dosing with fluoride. Browning of leaves appeared in random positions on all of the plants with no observations linked to the concentration of fluoride administered and leaves affected.

New growth was observed on all of the plants and the second fluoride dose was administered at Week 6. Plant growth continued to be observed from week 6, until week 12 at the experiment end. Leaves exhibiting browning continued to brown further, but mature leaves not exhibiting browning by week 6 did not subsequently, exhibit browning, Plate 6.1. However, on certain plants (at least one or more from each dosed group), new shoot growth appeared to be discoloured with browning and distorted growth, Plate 6.2.

After 12 weeks, prior to harvesting, the control group F showed no visible signs of browning leaves or shoot tips, whereas the 50, 100, 150 and 200 mg NaF dosed plants were observed to have at least 4 or more affected leaves. In addition 68% of the dosed plants exhibited damage to bud growth, similar to Plate 6.2.



Week 1



Week 3



Week 6



Week 9

Plate 6.1 Leaf browning on plants between week 1 and 9 of the experimental period.

The plants dosed with  $\text{NH}_4\text{F}$ , showed similar browning of leaf trends as the  $\text{NaF}$  dosed plants, including the randomised leaves that were affected and signs of discoloured new bud growth.

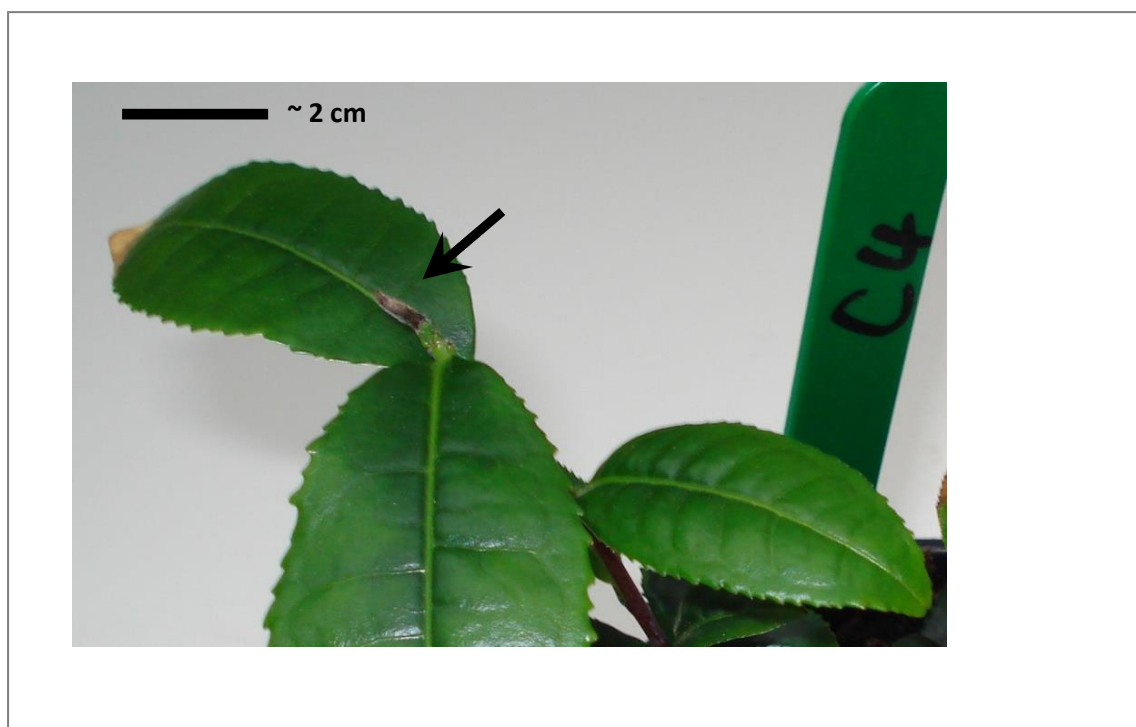


Plate 6.2 An example of 'browning' to the bud of tea plant

### 6.3.3 Fluoride distribution in the plant and soil

Fluoride concentrations for the plant parts (roots, bud and mature leaf) and total or water soluble fluoride in soil are summarised in Table 6.5. Total fluoride in both of the control soils F and G, ranged from 38 - 61  $\text{mgkg}^{-1}$ . However, when dosed, the total fluoride increased significantly ( $p < 0.001$ ) with a positive relationship ( $R^2 = 0.996$ ), in order of increasing fluoride addition, Figure 6.4. Water soluble fluoride concentrations in the soil control samples ranged from 0 - 10  $\text{mgkg}^{-1}$ . For total fluoride in the soils, a positive relationship was observed with increasing doses of fluoride, ( $R^2 = 0.957$ ), Figure 6.4.

For the buds of the tea plants, Table 6.5, the two control groups F and G, contained significantly lower fluoride concentrations ( $p<0.001$ ), ranging from 98 - 405  $\text{mgkg}^{-1}$ , compared to all the other NaF dosed groups, 445 - 4427  $\text{mgkg}^{-1}$ . In the mature leaves there were no significant differences between the fluoride doses with the exception of the 50 mg fluoride dose which was significantly higher ( $p<0.05$ ) than the other NaF doses. Total fluoride in the roots was not significantly different in concentration compared to the control samples, until the dose reached 150 mg, Table 6.5, where almost a 5 fold increase was observed.

Table 6.5 Mean fluoride concentration  $[\text{F}^-]$   $\text{mgkg}^{-1}$  in soil, plant buds, mature leaves and roots (mean  $\pm$  SD,  $n=3$ ) \* Dosed with  $\text{NH}_4\text{F}$

[F <sup>-</sup> ] dose	Group	Soil (total)	Soil (water)	Buds	Mature leaves	Roots
0 mg	G	47 $\pm$ 8	2 $\pm$ 2	121 $\pm$ 25	524 $\pm$ 33	217 $\pm$ 86
0 mg	F	53 $\pm$ 7	9 $\pm$ 1	358 $\pm$ 53	281 $\pm$ 35	298 $\pm$ 39
50 mg	A	719 $\pm$ 20	338 $\pm$ 9	2449 $\pm$ 82	3213 $\pm$ 239	217 $\pm$ 23
100 mg	B	1555 $\pm$ 47	706 $\pm$ 9	2548 $\pm$ 238	2274 $\pm$ 205	196 $\pm$ 19
150 mg	C	2115 $\pm$ 23	761 $\pm$ 23	3023 $\pm$ 288	2323 $\pm$ 209	931 $\pm$ 123
200 mg	E	3011 $\pm$ 120	1313 $\pm$ 69	1551 $\pm$ 89	3002 $\pm$ 69	1144 $\pm$ 20
150 mg*	D	2321 $\pm$ 104	1117 $\pm$ 38	5586 $\pm$ 95	3701 $\pm$ 746	1086 $\pm$ 142



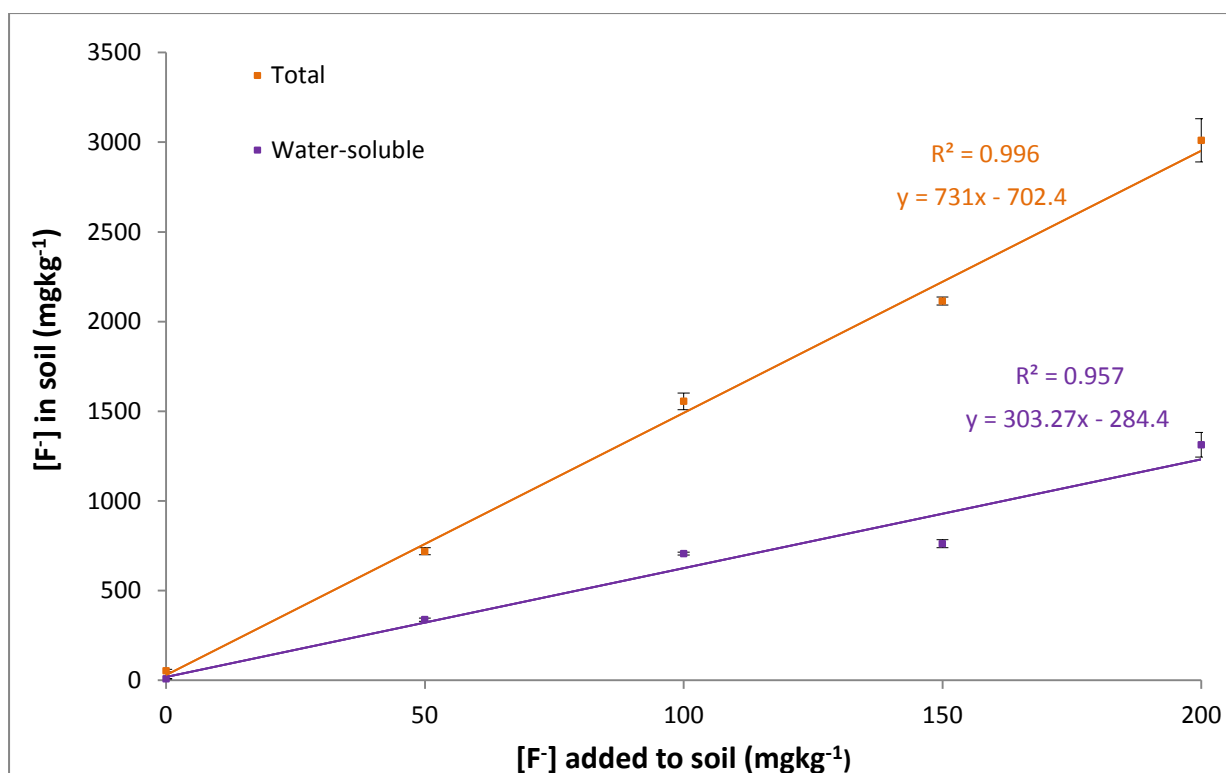


Figure 6.4 Mean fluoride concentration in soils with different fluoride doses as NaF (mean  $\pm$  SD,  $n = 3$ )

The buds dosed with fluoride from  $\text{NH}_4\text{F}$ , ranged from 5477 to 5654  $\text{mgkg}^{-1}$  and this was significantly higher in fluoride concentration compared to the other NaF dosed groups ( $p < 0.01$ ), Table 6.5. In the mature leaves, unlike the tea plant buds, there was no significant difference ( $p > 0.05$ ) in fluoride concentration between the  $\text{NH}_4\text{F}$  dose and the NaF doses. Fluoride in roots significantly increased with the dose of  $\text{NH}_4\text{F}$  compared to the other lower doses ( $p < 0.001$ ).

Total and water soluble fluoride concentrations in the soils were plotted against the fluoride concentrations in the roots, buds and mature leaves, Figure 6.5 and 6.6. For both total fluoride and water soluble fluoride in soil. The buds show a rapid decline in fluoride concentration when total fluoride in soil reached an excessive limit, where possibly the plants tolerance mechanism is failing (approximately 2,250  $\text{mgkg}^{-1}$  for

total fluoride, 1150 mgkg<sup>-1</sup> for water soluble fluoride). The mature leaves also show a reduction in fluoride at these soil concentrations, but not to the same extent.

There was no significant difference in the overall fluoride concentrations between buds and mature leaves ( $p>0.05$ ), but a lower concentration ( $p<0.05$ ) in the roots compared to the buds and mature leaves was observed. The coefficient of determination ( $R^2$ ) was calculated for each graph and in order of increasing positive relationship was roots > mature leaves > buds, similar for both total and water soluble fluoride in the soils.

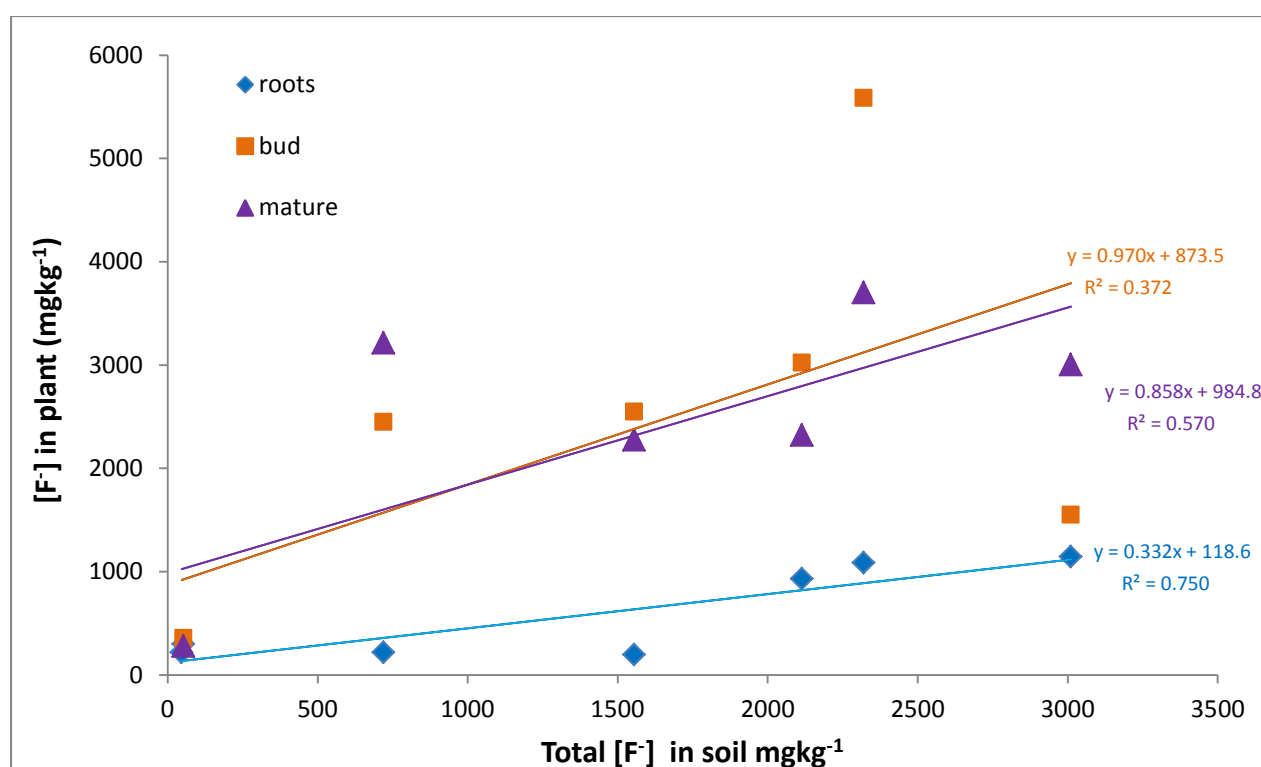


Figure 6.5 Mean total fluoride concentration in soil against mean fluoride in the roots, buds and mature leaves (mean  $\pm$  SD, n=3)

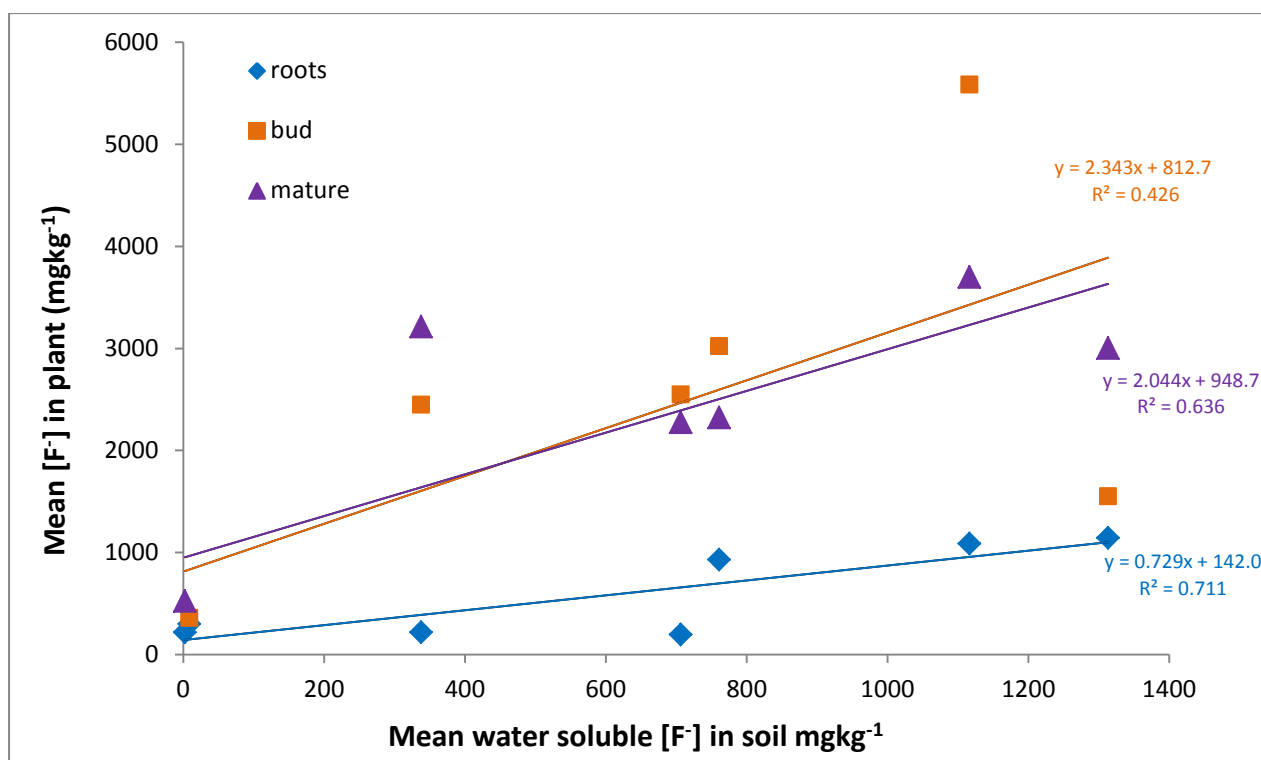


Figure 6.6 Mean water soluble fluoride concentration in soil against mean fluoride in roots, buds and mature leaves (mean  $\pm$  SD, n=3)

Figures 6.7 and 6.8 shows a comparison of fluoride dosing, either as NaF or as  $\text{NH}_4\text{F}$  where both concentrations of fluoride added to the soil were 150 mg. Fluoride concentrations were higher using  $\text{NH}_4\text{F}$  for the fluoride dosing rather than NaF in all sections of the plant (roots, buds and mature leaves) and in the soil, although there was no significant difference between the fluoride concentrations in the roots, Figure 6.7. A significant increase in fluoride concentration was observed in the buds and mature leaves of the  $\text{NH}_4\text{F}$  dose, ( $p < 0.05$ ). Total fluoride concentrations in soils were not significantly different ( $p > 0.05$ ), except  $\text{NH}_4\text{F}$  water soluble fluoride was statistically higher in concentration than NaF ( $p < 0.001$ ).

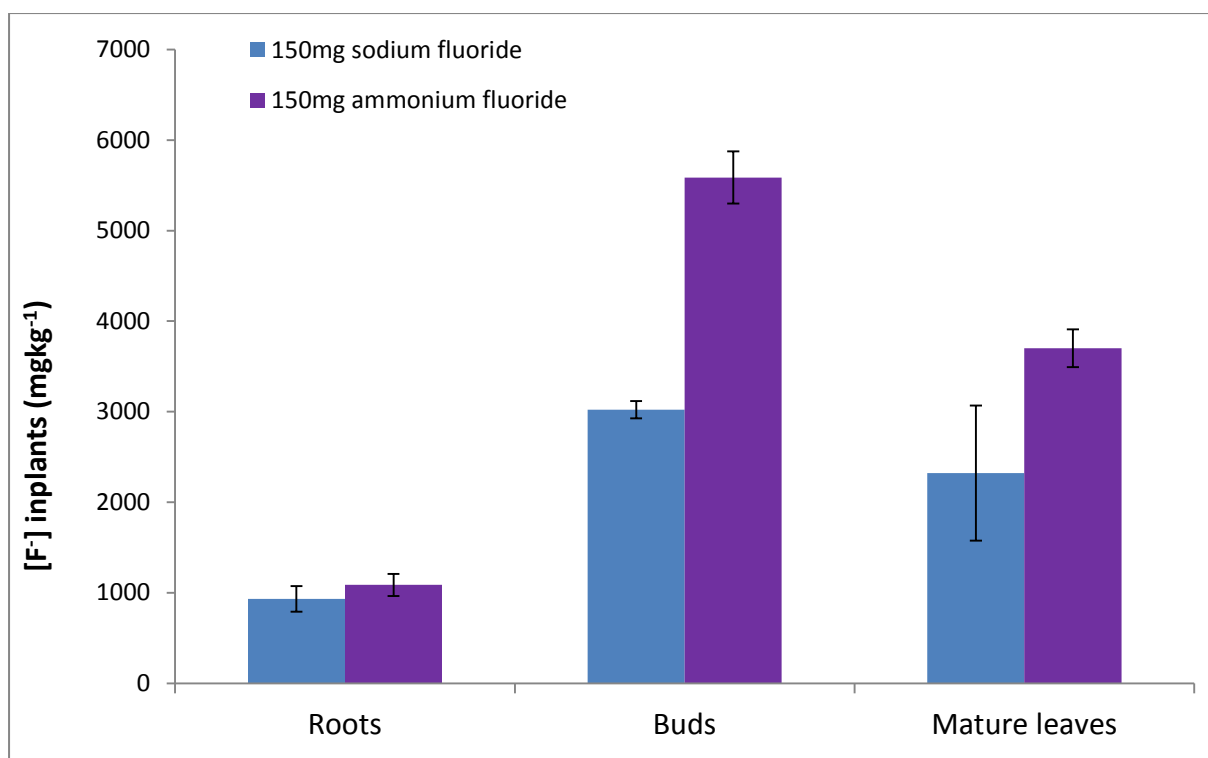


Figure 6.7 The effect of two different fluoride compounds of fluoride on plant fluoride distribution (mean  $\pm$  SD, n=3)

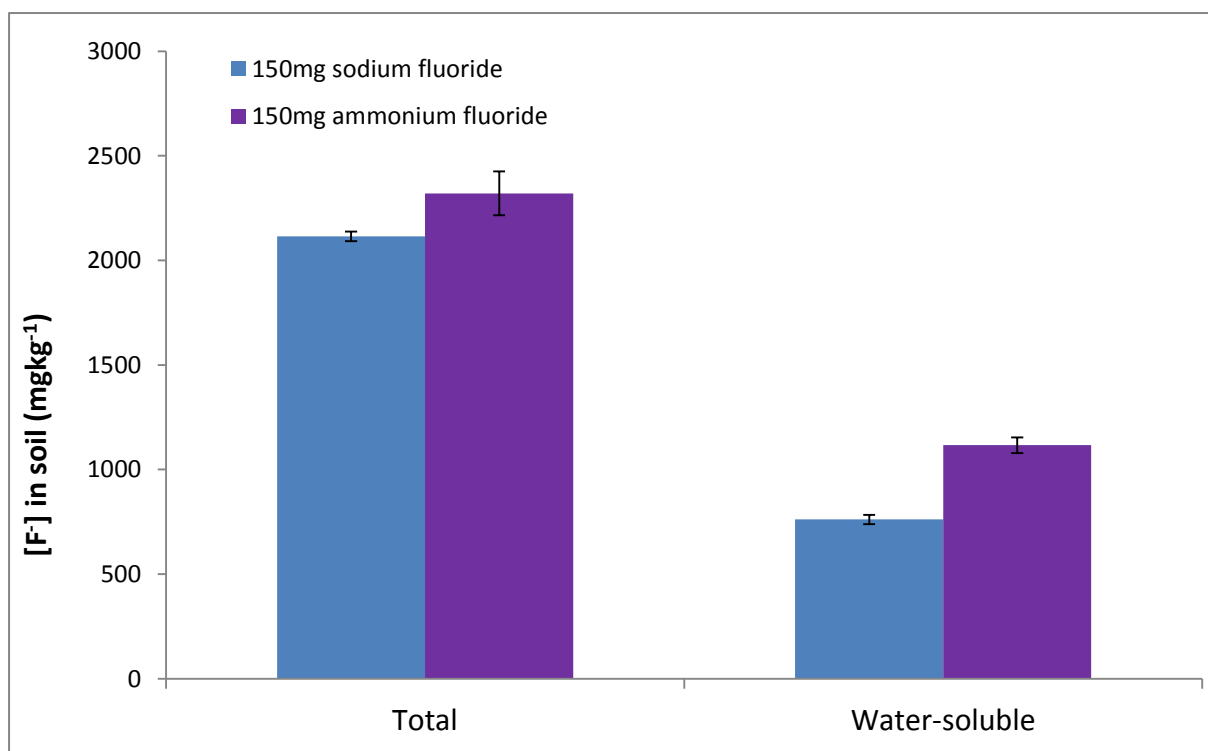


Figure 6.8 Fluoride concentrations in soil, using two different fluoride compounds (mean  $\pm$  SD, n=3)

The calculated transfer factor ratios from soil to the different parts of the plant from the roots to buds and mature leaves are shown in Figure 6.9 and 6.10. The higher transfer factors, from the soil to the rest of the plant, appears to be in the plants where no additional fluoride was added, ranging from 2.6 - 11.2, Figure 6.10. Transfer of fluoride to the parts of the plant for the 2 week control plants G were in the order of soil to mature leaves > soil to roots > soil to buds, but for the 12 week control plants F, the order was soil to buds > soil to roots > soil to mature leaves. The highest fluoride treatment 200 mg, displayed the lowest transfer factor of fluoride from the soil to buds and mature leaves, ranging from 0.5 - 1.0.

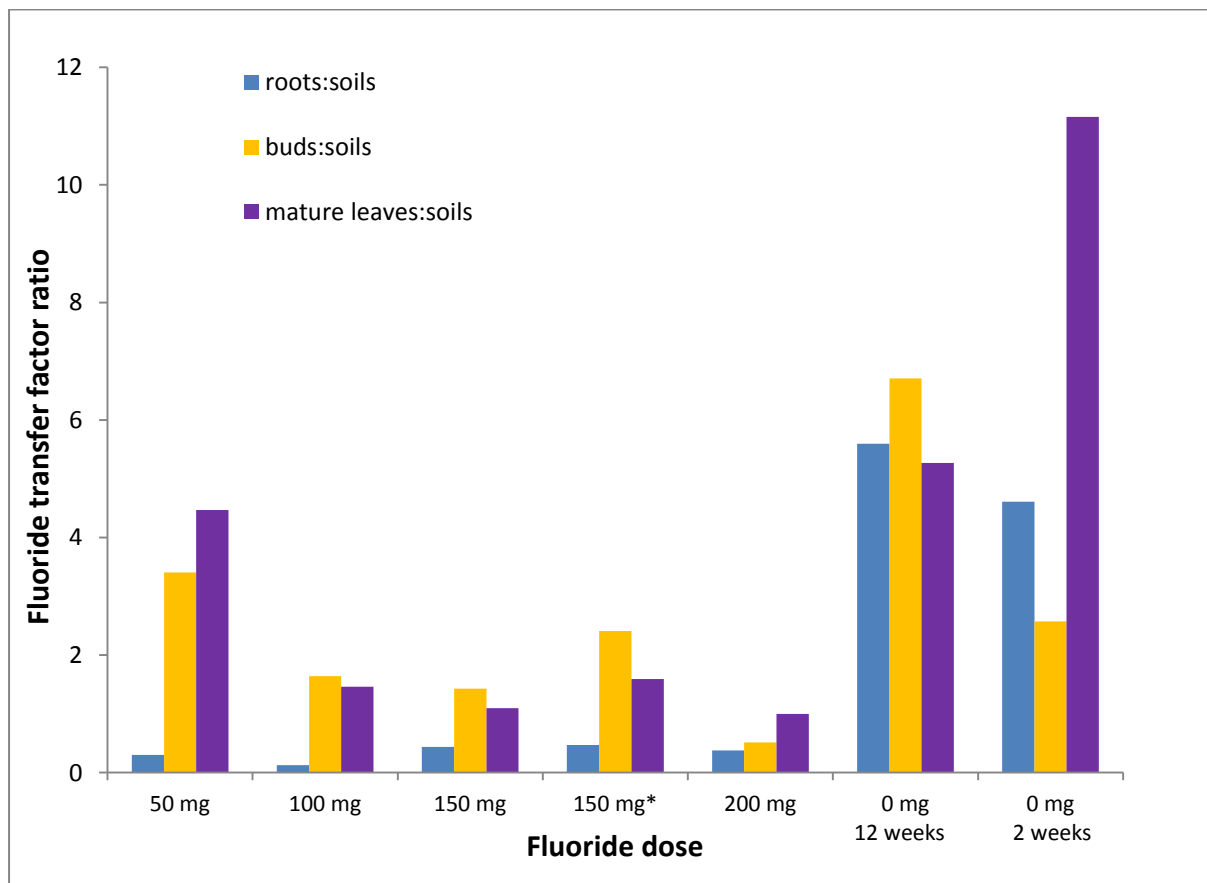


Figure 6.9 Transfer factor ratio of fluoride from soil to different parts of the plant

For the NaF dosed plants, the transfer factor of fluoride for 50 mg and 200 mg were in the order of soil to mature leaves > soil to buds > soil to roots. Dosed plants, 100 mg and 150 mg, followed the order of soil to buds > soil to mature leaves > soil to roots.

Transfer factors from the soil for the  $\text{NH}_4\text{F}$  plants were similar in values to the 100 mg and 150 mg NaF doses and in the same order as buds > mature leaves > roots.

Fluoride transfer from the roots to the buds and mature leaves in the NaF dosed plants appear to be the greatest in the lower doses of 50 mg and 100 mg fluoride, ranging from 11.3 - 14.8, Figure 6.10. In contrast the lowest transfer factors are observed for both of the control samples F and G, ranging from 0.6 - 2.4. The 50 mg treatment exhibits a transfer order of roots to mature leaves > roots to buds, similar to 200 mg and 0 mg (control G), whereas an order of roots to buds > roots mature leaves is observed for 100 mg, similar to 150 mg and 0 mg (control F).

Transfer factors for the  $\text{NH}_4\text{F}$  dosed plants were in the order of roots to buds > roots to mature leaves and were higher than all of the NaF dosed plants.

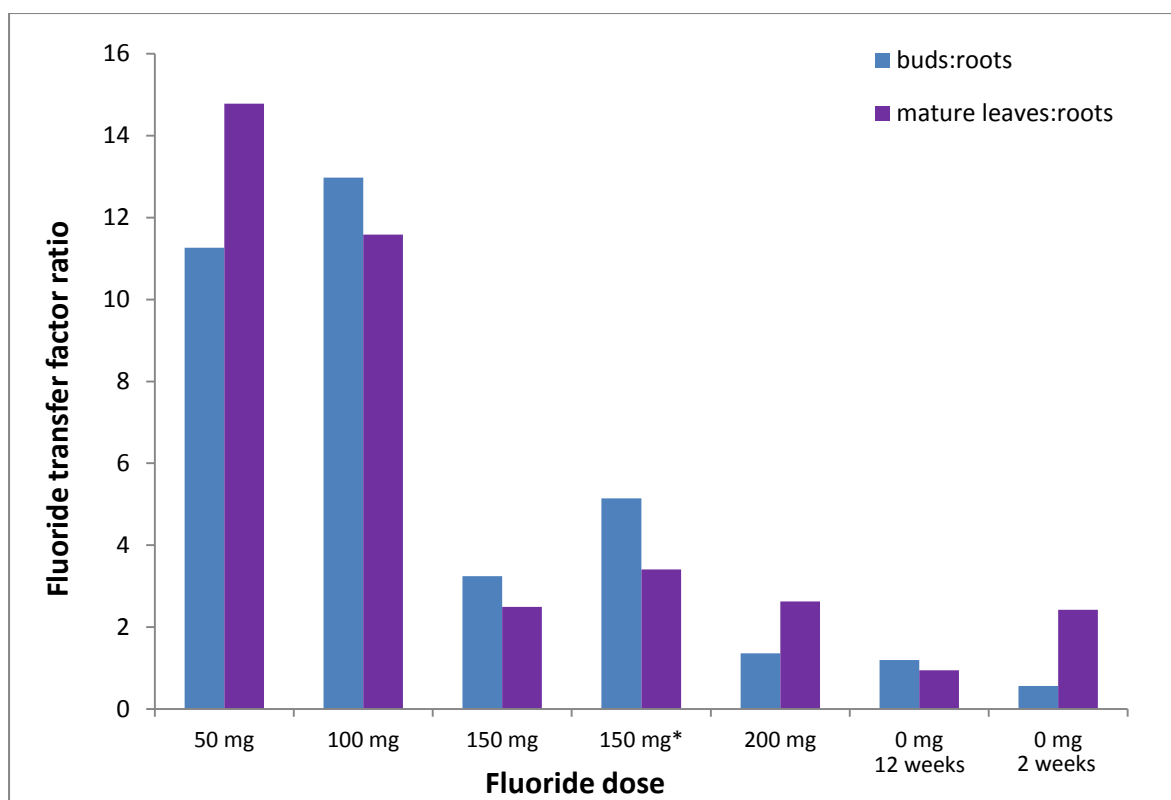


Figure 6.10 Transfer factor ratio of fluoride from the roots to the buds and mature leaves

### 6.3.4 Quality control

Background fluoride concentrations were determined by harvesting untreated plants at 2 weeks into the experiment after initial re-potting and by treating the control group F in the same way as the dosed plants; growing and harvesting with the dosed plant groups. Figure 6.11 is a comparison of fluoride concentrations determined in the roots, buds and mature leaves of both control samples.

Fluoride concentration is not significantly higher in the untreated 12 week experimental control roots and buds, ranging from 262 - 339 mgkg<sup>-1</sup> and 301 - 405 mgkg<sup>-1</sup>, respectively, compared to the 2 week background control, ranging from 25 - 268 mgkg<sup>-1</sup> and 98 - 282 mgkg<sup>-1</sup>, respectively ( $p>0.05$ ). The 2 week control has a

significantly higher concentration of fluoride in the mature leaves compared to the 12 week control, ranging from 543 - 733  $\text{mgkg}^{-1}$  and 164 - 261  $\text{mgkg}^{-1}$ , respectively ( $p < 0.05$ ).

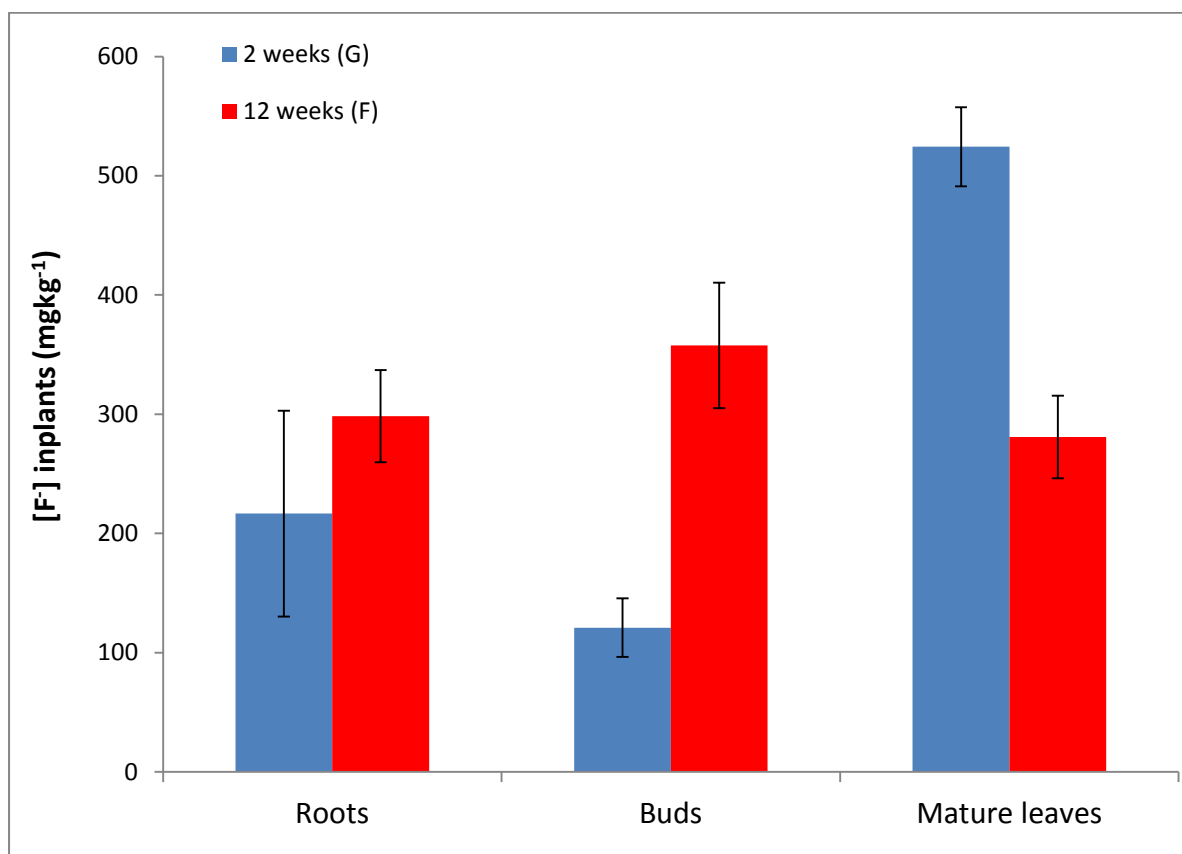


Figure 6.11 Mean fluoride distribution in the control plant soils, F and G (mean  $\pm$  SD,  $n=3$ )

Figure 6.12 shows a comparison of fluoride concentrations in the soils of both control groups. Overall total fluoride ranged from 38 - 61  $\text{mgkg}^{-1}$  for the controls with water soluble fluoride, ranging from 0 - 10  $\text{mgkg}^{-1}$ . The soil of the untreated control plants at 12 weeks contained significantly higher water-soluble fluoride concentrations than those at 2 weeks ( $p < 0.05$ ), but was not statistically different for total fluoride ( $p > 0.05$ ).



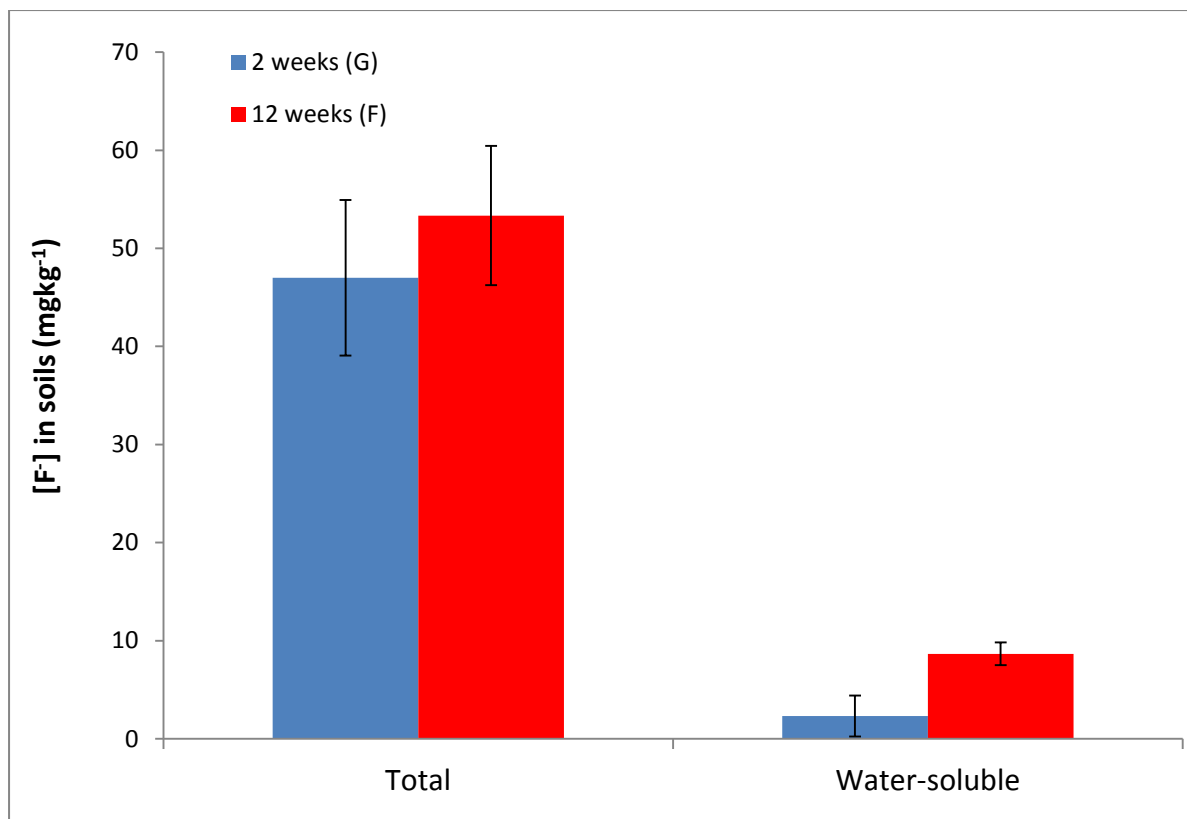


Figure 6.12 Mean fluoride concentration in the control plant soils, F and G (mean  $\pm$  SD, n=3)

Analytical fluoride recoveries, to support the concentrations determined in the soils and plants are given in Table 6.6. Results for the tea and soil CRM's were within the certified values with mean recoveries no greater than 110%. Mean fluoride concentration in the blank was minimal at  $0.2 \text{ mg l}^{-1}$  and standard recoveries were  $\geq 98.3\%$

Table 6.6 Determined fluoride concentrations ( $\text{mgkg}^{-1}$  or  $\text{mg l}^{-1}$ ) for CRM, standards and blanks

Sample	n	Certified value [F <sup>-</sup> ]	Determined mean and SD [F <sup>-</sup> ]	% Mean recovery
Tea CRM GBW10016	9	$57 \pm 15 \text{ mgkg}^{-1}$	$63 \pm 7 \text{ mgkg}^{-1}$	110%
Soil CRM NCS ZC73006	3	$652 \pm 48 \text{ mgkg}^{-1}$	$675 \pm 3 \text{ mgkg}^{-1}$	103%
Blank	9	n/a	$0.2 \pm 0.1 \text{ mg l}^{-1}$	n/a
5 $\text{mg l}^{-1}$ standard	1	n/a	$4.94 \text{ mg l}^{-1}$	99.6%
15 $\text{mg l}^{-1}$ standard	1	n/a	$14.74 \text{ mg l}^{-1}$	98.3%
25 $\text{mg l}^{-1}$ standard	2	n/a	$25.09 \text{ mg l}^{-1}$	100.4%

## 6.4 Discussion

With the control group exhibiting an extremely significant increase in plant growth suggests that additional fluoride in the soil inhibits plant growth. This is supported by the  $R^2$  coefficient, Table 6.2, where the control plants F, display a high positive growth relationship with dose dependency of  $R^2 = 0.994$  compared to the highest fluoride dose of 200 mg with the lowest  $R^2 = 0.395$ . A study by Jha *et al.* (2009) found a significant reduction in the biomass of the roots, shoots and bulb of the onion (*Allium cepa* L.) with higher doses of fluoride. Decreases in biomass of 20%, 59% and 70% were reported for doses 400, 600 and 800  $\text{mgkg}^{-1}$  NaF, respectively (Jha *et al.* 2009). Jha *et al.* (2008) also studied the response of fluoride additions to spinach

(*Spinacea oleracea*) and in alkaline soil and the biomass significantly decreased ( $p>0.05$ ) with doses of 600 and 800  $\text{mgkg}^{-1}$  of NaF. It should be noted that Jha *et al.* (2008, 2009) dosed their plants with concentrations of NaF, whereas in this study the dose represented fluoride only from the compound NaF, so a dose of 800 mg NaF  $\text{mgkg}^{-1}$  in Jha *et al.* (2008, 2009) study would be the equivalent of approximately 362  $\text{mgkg}^{-1}$  of fluoride in the present study.

Although biomass was not evaluated in this study, at the 12 week period, a percentage decrease in plant growth ranged from 68.8 - 86.6%, for the dosed plants compared to the control group F, which is similar to Jha *et al.* (2008, 2009) studies that plant mass decreases when the plants are exposed to additional fluoride. In contrast to the findings in this study, Ruan *et al.* (2004) observed no difference in plant dry matter of *C. sinensis* seedlings, from fluoride applications in soil of up to 100  $\text{mgkg}^{-1}$  as  $\text{NH}_4\text{F}$ . Ruan *et al.* (2004) focused on the effect of liming soil with the addition of calcium oxide (CaO), making comparisons to this study difficult.

Visible toxicity, in the form of leaf tip browning, was observed in all of the plants exposed to fluoride dosing, despite the concentration administered. A distinct indication of fluoride toxicity to plants is browning of the leaf tips and edges, 'tip burn'. Literature describing leaf browning from fluoride toxicity is limited to plants that appear to be sensitive to fluoride, such as *Hypericum perforatum*, *Cordyline terminalis*, *Chrysanthemum* and *Antirrhinum* (Marousky and Woltz 1975; Elliott 1982; Fornasiero 2001; Campos *et al.* 2010). Although literature was not found describing leaf browning in *C. sinensis*, the necrosis observed compared to the description of fluoride toxicity described by Fornasiero (2001) from uptake of sodium fluoride through roots of the *H. perforatum* as, "marginal necrosis, separated from the healthy, unaffected tissues by a sharply marked line." Plate 6.3 shows the leaf burning and line separating the unaffected part of the leaf, from a plant dosed with 50 mg of fluoride, at 7 weeks into the experiment. In addition, the 12 week

experimental control plants showed no sign of the ‘tip burn’ necrosis, which supports the observed fluoride toxicity of the dosed plants in this study.



Plate 6.3 Marginal necrosis ‘tip burn’ with unaffected area of leaf.

Following the two scheduled fluoride doses, during week 8 and 12, inhibition of the mature leaf growth was observed for Group E (200 mg), Figure 6.3. Although this may suggest that excess fluoride inhibits the growth of mature leaves at such concentrations, the visual effects observed of leaf browning or necrosis may have affected the leaves, which were measured, therefore interfering with a healthy leaf measurement. The browning observed in the mature leaves could be the result of the plants’ survival mechanism, by isolating the excess fluoride to certain parts of the leaf eventually this leads to cell necrosis of that area. Leaf browning observed is a result of pigment breakdown and is a sign of cell necrosis. By adopting this isolating mechanism, the remaining green area of the leaf can continue to photosynthesise.

With the wide variation in World background fluoride soil concentrations ( $<10 - 1360 \text{ mgkg}^{-1}$ ), total fluoride in the control soils,  $50 \pm 8 \text{ mgkg}^{-1}$  (mean and SD,  $n = 6$ ) were relatively low compared to the ranges of  $113 - 580 \text{ mgkg}^{-1}$  in Great Britain, and  $260 - 520 \text{ mgkg}^{-1}$  in Japan (Kabata-Pendias 2010). Tea plantations of Lantau Island, China are reported to have total fluoride concentrations ranging from  $299 - 371 \text{ mgkg}^{-1}$  which are within the natural backgrounds concentrations for the UK and Japan (Fung *et al.* 2003). In contrast water soluble fluoride concentrations in the control soils,  $5.5 \pm 3.8 \text{ mgkg}^{-1}$  (mean and SD,  $n = 6$ ), were slightly elevated compared to those reported in literature. Fung *et al.* (2003) reported a range of  $1.4 - 2.6 \text{ mgkg}^{-1}$  and Ruan and Wong (2001) reported a range of  $0.1 - 2.5 \text{ mgkg}^{-1}$  in soils from typical tea plantations in China. The soil used for the experiment was a special mix of ericaceous compost; however this was used throughout the study and was considered as a viable medium. In addition, many studies on fluoride uptake have involved the use of hydroponic systems (Stevens *et al.* 1997; Mackowiak *et al.* 2003; Li and Ni 2009; Zhang *et al.* 2013) or were carried out using tea plantation soils or contaminated soils (Wenzel and Blum 1992; Arnesen 1997; Fung *et al.* 2003; Shu *et al.* 2003; Xie *et al.* 2007) and this was not possible to replicate.

Hydroponic systems are very different to soil systems, as the natural environment is not replicated, where pests or disease can be eliminated and the exact nutrients required for a certain species of plant can be provided (Mason 2000). These systems are more suitable for soft fruits and vegetables, such as tomato, strawberry and lettuce where quick growth and high fruit yield is required (Mason 2000). Although a soil system carried out in a glass-house is not completely typical either, it can mimic the natural chemistry of soil, including mineral or metal adsorption/desorption, ion exchange and redox reactions (Sparks 2003). This was thought to be more representative to a typical tea plantation and therefore adopted for this study.

The bud of *C. sinensis* is well documented to contain the least fluoride in comparison to the other leaves of the bush (Shu *et al.* 2003). However, all of the dosed plants and control sample F had elevated fluoride concentrations in the buds compared to literature, Table 6.5 (Shu *et al.* 2003). However, the control plants compared fairly well to literature for fluoride concentration in the buds, with Shu *et al.* (2003) reporting a range of 54 - 181 mgkg<sup>-1</sup> and 49 - 602 mgkg<sup>-1</sup> was determined by Xie *et al.* (2007). Contributing factors to the excess fluoride in the buds of the dosed plants may have been the juvenility of the plants used in this experiment and the form and concentration of fluoride administered to the plants, which was not completely typical to a natural system. Fluoride in the form of NaF is readily soluble, which allows it to become mobile in soil water and concentrations administered were taken to the extreme to see how much fluoride *C. sinensis* is capable of accumulating. Another main observation was the significant increase of fluoride concentration using NH<sub>4</sub>F compared to NaF in buds at the same dosage concentration of 150 mg. The main difference was the ammonium ion (NH<sub>4</sub><sup>+</sup>) in NH<sub>4</sub>F, which is readily absorbed by plants for nitrogen to synthesise essential amino acids (Harman 2006). This factor could contribute to the fluoride increase in the younger leaves as the plant was able to readily absorb the fluoride in this form to the younger tissues in need to synthesise amino acids for growth. In addition, NH<sub>4</sub>F is far more soluble than NaF, being 45.3 g compared to 4.3 g per 100 ml of water at 25°C (Haynes 2013), which may lead to increased water soluble fluoride for uptake by the plant in the NH<sub>4</sub>F form.

As previously established, fluoride accumulates in the mature leaves of *C. sinensis*. This study agreed with literature, showing elevated fluoride concentrations in mature leaves (Fung *et al.* 1999; Fung *et al.* 2003; Ma *et al.* 2002; Shu *et al.* 2003; Xie *et al.* 2007) with the control groups F and G, ranging from 164 - 733 mgkg<sup>-1</sup>, and from 1439 - 4523 mgkg<sup>-1</sup> for the other dosed groups A to E. The fluoride concentrations in the controls of this study show a fairly wide range, but are similar to Xie *et al.* (2007)

study, reporting concentrations from 221 - 1504 mgkg<sup>-1</sup> for *C. sinensis* grown in plantations in central and southwest China. In contrast, other authors state wider ranges for *C. sinensis* growing in plantations. Shu *et al.* (2003), reported a range of 228 - 2965 mgkg<sup>-1</sup> and Fung *et al.* (2003) published a range of 1350 - 2900 mgkg<sup>-1</sup>, in which the fluoride dosed plants from this study are within the lower range, but exceed the higher end.

The roots of the control samples F and G contained a fluoride concentration range of 117 - 339 mgkg<sup>-1</sup> which was in agreement with those published by Fung *et al.* (2003) as 250 - 380 mgkg<sup>-1</sup>, but elevated compared to Shu *et al.* (2003), ranging from 19 - 69 mgkg<sup>-1</sup>. In the dosed plants  $\geq 150$  mg of fluoride, the behaviour of excess fluoride storage in the root system was not apparent in any current literature; however, this is typical behaviour for other species of plants exposed to excess fluoride. According to Stevens *et al.* (2000), species of *Trifolium subterranean*, *Dactylis glomerata*, *Hordeum leporinum*, *Onopordom acanthium* and *Rumex acetosella* exhibited significant increases ( $p < 0.05$ ) of fluoride in the root systems, after concentrations of 0.94 mM ( $\sim 18$  mg l<sup>-1</sup>) fluoride in nutrient growing solutions, for *D. glomerata* and *H. leporinum* and 1.5 mM ( $\sim 28$  mg l<sup>-1</sup>) fluoride for the other plants. In this study, the higher doses of fluoride may have meant that excess fluoride was stored in the root system of *C. sinensis*. In addition, a significant decrease in fluoride ( $p < 0.01$ ) was observed for the highest fluoride dose (E) at 200 mg in the buds, which may attribute to using the roots for further storage.

A positive relationship for the coefficient of determination ( $R^2$ ) was observed, ranging from 0.426 to 0.711, for fluoride uptake into the *C. sinensis* buds, mature leaves and roots, with increasing water soluble fluoride concentrations, Figure 6.6. Ruan *et al.* (2003) reported a linear correlation from external fluoride and its uptake of fluoride as  $R^2 = 0.88$  ( $n=3$ ) from soil, so in agreement, this study also showed a positive a correlation. Water soluble fluoride is reported to be a better indicator of

fluoride availability, because of the variation in soils natural sorption capacity (Arneson 1997). Soil pH is a contributing factor to absorption of fluoride as previously mentioned and the pH of the soils were all acidic pH <5.1. Arneson (1997) and Ruan *et al.* (2004) report that by altering the acidity of soil to a more neutral soil will significantly reduce the uptake of fluoride from soil. With the acidic soils in this study, fluoride would form aluminium-fluoride complexes in the soil (Wenzel and Blum 1992; Fung and Wong 2002). However, the doses in this experiment were added in the form of NaF, and sodium (Na) is less likely to bind to soil (Arneson 1997), leaving the fluoride anion available for uptake by the roots through the xylem to reach the other parts of the plant (Zhang *et al.* 2013). The reason why *C. sinensis* allows a passage of fluoride through its roots and other species of plants inhibit this process is not completely understood. Zhang *et al.* (2013) suggest the strong capability of the tea plant to absorb fluoride is due to an energy dependent anion transport system which is not typical in non fluoride accumulating plants such as barley, tomato and rice (Bar-Yosef and Rosenberg 1988; Stevens *et al.* 2000; Weinstein and Davidson 2004; Zhang *et al.* 2013).

The transfer factor of fluoride distribution to the plant buds, leaves and roots from the soil did not form any obvious similarities in this study, however notable differences were observed between the control samples. The 2 week control favoured transfer to the mature leaves, whereas the control of 12 weeks favoured transfer to the buds. The plants arrived from the Tregnothnan tea estate in 'Camelia special mix' but the time when they were last re-potted was not known. Re-potting was carried out to ensure uniformity, but increased soil to plant interactions may have occurred as the 12 week control became more established in the 'Camellia special mix' potting compost, whereas the other control was harvested after 2 weeks. Both the control transfer factors were higher compared to the NaF dosed plants which indicated that additional fluoride in soil affects the efficiency of



transfer. To enhance this theory, an overall decrease of transfer ratio from the soil to plant was observed with increasing fluoride doses.

Ratios for the transfer of fluoride from the roots to either the mature leaves or buds were the lowest in the control samples which would be expected in natural conditions, as the roots did not contain excess fluoride. With the lower doses of NaF, A, 50 mg and B, 100 mg, the transfer ratio's were significantly higher than all of the other doses ( $p < 0.001$ ). This indicates *C. sinensis* favours storage of fluoride in the leafy parts of the plant over the roots. However, when fluoride reaches a certain concentration in the soil (>150 mg in this study), the transfer rate was dramatically reduced, suggesting a possible mechanism of *C. sinensis* preventing excess fluoride entering the xylem (Huynh *et al.* 2006).

When evaluating the uptake of increasing fluoride from soil and the accumulation in the plant, Figure 6.5, it appears that *C. sinensis* favours fluoride accumulation in the mature and younger leaves from soil concentrations of 0 - 800 mgkg<sup>-1</sup>. Above this soil concentration range, up to approximately 2100 mgkg<sup>-1</sup>, the mature leaves appear to adopt an exclusion mechanism and a slight reduction in fluoride accumulation is observed, whereas the buds continue to accumulate fluoride. This may relate to the first visual effects of mature leaf browning, Plate 6.2. For the concentration range of 0 - 1500 mgkg<sup>-1</sup>, the roots appear to be excluding fluoride, thus removing the excess rapidly into the leaved areas of the plant. Above this range, the roots start to accumulate the excess fluoride, which could be the reason for the decrease observed in the mature leaves. The root to mature leaves or buds transfer mechanism appears to breakdown at extreme soil fluoride concentrations above 2100 mgkg<sup>-1</sup> where a dramatic accumulation of fluoride appears to favour the buds, over the accumulation in the mature leaves. At 2600 mgkg<sup>-1</sup>, the plants may have become completely saturated with fluoride, and a rapid decline in fluoride accumulation is observed,

more so in the buds. This relates to the visual effects observed of browning to the buds, where necrosis is occurring.

Although it would not be typical to have soil fluoride concentrations as high as used in this study, it was interesting to observe the effects in *C. sinensis*, especially visual observations of leaf browning due to fluoride toxicity and the changes in transfer ratios throughout the plant with varying doses. However, despite the excess fluoride administered, growth continued, enhancing present literature of the fact that *C. sinensis* is a fluoride accumulator. Even the young buds of the plants can contain elevated concentrations of fluoride, which are used to manufacture the tea beverage. However, it should be noted that the plants used in this study were cuttings of approximately 2 years old, so it was difficult to obtain representative masses of plant material for analyses which could have caused certain limitations in the data.

## 6.5 Chapter Summary

- After additions of fluoride concentrations  $\geq 50\text{mgkg}^{-1}$  to soil, *C. sinensis* exhibited signs of leaf browning and marginal necrosis in certain leaves.
- *C. sinensis* can tolerate high fluoride concentrations in soil, up to  $3011\text{mgkg}^{-1}$  of fluoride with continual growth, but the rate of growth decreased significantly compared to the control plants.
- Fluoride concentrations in the dosed tea plants were mostly in the order of mature leaves > buds > roots, agreeing with current literature of accumulation in the mature leaves. However, in a few cases, the buds exhibited elevated fluoride accumulation not typical to published literature.

- In the form of  $\text{NH}_4\text{F}$  fluoride concentration was significantly higher in the buds compared to dosing the plant with NaF.
- An increase in fluoride storage of the root systems was observed when *C. sinensis* was dosed with NaF concentrations  $\geq 150 \text{ mgkg}^{-1}$ , and this appeared to be related to a possible breakdown in the accumulation mechanism of fluoride in the mature leaves at excess concentrations.

## **Chapter 7: General Discussion**

## 7.1 Introduction

The WHO recommends a concentration of  $1.5 \text{ mg l}^{-1}$  fluoride in drinking water, in order not to cause any risk to human health over a life time. However, WHO also state that concentrations exceeding this carry an increased risk of dental and skeletal fluorosis (WHO 2004). Fluoride is an essential mineral in the human diet, at an optimum concentration. Therefore, it is important to know the concentrations of fluoride in tea and to consider tea as a vehicle of fluoride dosing in the diet, in relation to possible health issues. Determining fluoride concentrations in tea products and fluoride leaching into the infusion can establish the transfer of fluoride during the tea brewing process. Furthermore, by developing an *in vitro* bioaccessibility technique, it can be estimated whether fluoride from a tea infusion is available for absorption by the human gut.

## 7.2 Method development for fluoride determination in tea products, infusions, plants and soils

Successful determination of total fluoride in tea products, tea plants and soils was accomplished using alkali fusion and IC instrumentation. Accurate percentage fluoride recoveries of the CRM's ranging from 99 - 100% were achieved using this methodology. IC was chosen as a modern technique which can be automated to run a large number of samples (Michalski 2006). The limit of detection for fluoride concentration ( $0.1 \text{ mg l}^{-1}$ ) was suitable for the range in this study, from 98 - 5653  $\text{mg kg}^{-1}$ . However, it should be highlighted that the method developed in this study was unique by using a liquid form of 50% (w/v) potassium hydroxide for the fusion rather than solid potassium hydroxide reported by Metrohm (2008). The aqueous potassium hydroxide provided a more uniform sample slurry mixture for the fusion

to occur compared to using dry solid potassium hydroxide. A similar method by Sparks *et al.* (1996), involves the use of aqueous sodium hydroxide, but the detection describes the use of ISE (Fung *et al.* 1999; Malde *et al.* 2001; Shu *et al.* 2003). Aqueous potassium hydroxide was chosen as a modification from sodium hydroxide to incorporate the choice of instrumentation as it ensured that the sample was at the correct pH to prevent chromatographic column damage.

When using IC to determine fluoride in the tea infusions, interference occurred, as the sample matrix was not broken down. Interfering compounds, such as acetate or formate, affected the fluoride peak separation (Janiszewska and Balcerzak 2013). Therefore, methods described in existing literature were followed using ISE for detection (Duckworth and Duckworth 1978; Effendi and Wibowo 1985; Smid and Kruger 1985; Schamschula *et al.* 1988; Gulati *et al.* 1993; Chan and Koh 1996; Cao *et al.* 2004; Chandrajith *et al.* 2007; Sofuoglu and Kavcar 2008; Malinowska *et al.* 2008; Yi and Cao 2008; Koblar *et al.* 2012; Quock *et al.* 2012). Fluoride concentrations in the tea infusions were lower compared to total fluoride in the tea products and over a much smaller range (0.4 - 8.8 mg l<sup>-1</sup>), measured and reported as the ion present in the liquid matrix. ISE was decidedly more suitable for this range, as this technique was calculated to have a lower limit of detection compared to IC, of 0.03 mg l<sup>-1</sup>. This method also eliminated any organic interferences observed when trying the IC methodology (Michalski 2006). Accurate percentage fluoride recoveries of the CRM were achieved, ranging from 93 - 114%.

The study used two approaches to determine fluoride concentrations, as discussed previously. The compatibility of the two instrumentation techniques was necessary and compared by carrying out regular standard checks of ISE using the standard solution used for the IC calibration. The 3 mg l<sup>-1</sup> and 5 mg l<sup>-1</sup> standards used for IC, produced results of 2.9 ± 0.1 mg l<sup>-1</sup> and 4.9 ± 0.2 mg l<sup>-1</sup> using ISE, respectively.

### 7.3 Fluoride dosing and its uptake and distribution in tea plants

The experimental *C. sinensis* plants were dosed with concentrations of fluoride, ranging from 50 - 200 mg. In order to exceed normal background soil concentrations, an ionic form of fluoride was chosen, administered directly into the individual plant pots (Kabata-Pendias 2010; Edmunds and Smedley 2013). The form of fluoride and the concentrations were selected to investigate the plants tolerance to fluoride and to identify any mechanism involved in the uptake and distribution. Within four weeks a visual response was observed for all of the dosed plants, showing signs of browning leaf necrosis, compared to the unchanged leaves of the control plants. However, despite the high doses of fluoride administered, the plants continued to grow with signs of new shoots in random positions.

When the buds, mature leaves, roots and soils were analysed for fluoride, a positive correlation was observed for concentrations in the soil and uptake into the leaves for all of the dosed plants, except the control plants. This was backed up by the transfer ratios of fluoride from soil to the aerial parts, with the higher rates being within the dosed plants. This established the plants ability for fluoride uptake from the soil through the roots with further transportation to the aerial parts of the plant (Fung and Wong 2002; Shu *et al.* 2003).

The buds accumulated fluoride up to  $3023 \pm 288 \text{ mgkg}^{-1}$  similar to the mature leaves of up to  $3213 \pm 239 \text{ mgkg}^{-1}$ . The buds also showed signs of browning, typical of fluoride toxicity which may be due to the solubility and high doses administered to the plants (Fornasiero 2001). Typically, a mature *C. sinensis* plant would usually contain significantly less fluoride in the buds, ranging from 54 -  $181 \text{ mgkg}^{-1}$  compared to up to 2893 in the mature leaves (Shu *et al.* 2003). Despite this, fluoride

accumulation was observed in the mature leaves at concentrations similar to those reported by Shu *et al.* (2003).

The buds and leaves of *C. sinensis* form the main raw material for the production of dry tea products. However the type of leaves used depends upon the product. The finest quality tea uses the buds only, whereas a cheaper tea will include mature leaves (Yorkshire Tea 2013). Therefore, the parts of the plant used in the manufacturing process reflect the concentrations of fluoride present in the product. By controlling which part of the plant is used in tea manufacture, a reduction in fluoride concentrations in tea products could be achieved.

## **7.4 Fluoride concentrations in tea products**

From the thirty eight tea products analysed, the range of fluoride concentrations varied significantly, from 103 to 839 mgkg<sup>-1</sup> and in the order of Economy blends > Green blends > Black blends > Oolong/Pu'er > Pure blends. Economy blends are strictly Black blends, but were placed into separate groups after initial analyses reflected the difference from the other Black blends in terms of their high concentrations of fluoride. The other groups are all different types of tea based on the manufacturing process with the exception of the Pure blends, which are from specific geographical locations, such as Sri Lanka.

From the labelled groups, Economy blends, Black blends and Pure blends all undergo the same black tea manufacturing process, involving natural fermentation, followed by complete oxidisation, rolling and drying (Gascoyne *et al.* 2011). Oolong is only partially oxidised and Pu'er is fermented and sun dried. Green blends, on the other hand are not fermented or oxidised, but are pan fired immediately, to prevent natural fermentation from occurring (Pettigrew and Richards 2008). Fluoride concentrations in these different teas are not controlled or do not relate to the



manufacturing processes. Therefore, this study indicated that it is the part of the tea plant used, which is the key factor involved in determining how much fluoride is in the tea product.

Speciality teas Oolong and Pu'er are the most expensive products in the UK market and are normally only available from specialist tea shops. Pure blends, such as Assam, Darjeeling and Ceylon are widely available with prices from approximately £1.80/100g. Prices for Green blends and Black blends are similar and range from approximately £0.80 to £1.10/100g. As the name suggests, Economy blends are the least expensive at approximately £0.11/100g (pricing at August 2013). Mature leaves, are in abundance on a tea bush compared to the buds and by including these in a product could reduce the raw material costs. In addition to keeping the production costs down, the plucking techniques may be coarse or involve machinery which can lead to the inclusion of mature leaves (Owuor *et al.* 2011).

Tea manufacturers state that the improved quality of a tea product is dependent upon the part of the tea plant used (PG Tips 2013; Yorkshire Tea 2013). Silver Tips tea is one of the highest quality teas available using only the bud from the tea plant, whereas UK brand PG Tips, state the use of the top two leaves and the bud in the manufacture of the product (Saberri 2010; Tetley 2013). Pure blends may have strict plucking techniques to ensure only the buds are used, also these tea only include blends from a single origin as their name suggest, such as Assam or Darjeeling in India. In contrast, Black and Green teas can be sourced from many different countries, such as China, India, Kenya and Sri Lanka, and this may affect the quality (Samarasingham 2009; Tea Council 2013a; PG Tips 2013).

Brick tea uses mature leaves and can contain 200 - 300 times more fluoride than ordinary leaf teas (Cao *et al.* 2005). Based on the results for fluoride concentration in the tea products in this study, it could be assumed that the Economy teas are similar

to Brick tea, using mature leaves in the production, which allows these teas to be priced inexpensively. Although, a Waitrose tea was labelled as an economy product, fluoride concentration in this tea was significantly lower compared to the other Economy teas. The Waitrose tea was similar to the Black blends group. This may relate to the fact that Waitrose is positioned as a top-end supermarket and include quality produce within their 'Essential' economy range (Naylor 2010; Baker 2011), hence the possible differences in their tea manufacture compared to the other main supermarkets.

## 7.5 Fluoride in tea infusions

Tea products contain a substantial source of fluoride, but the concentration in the tea infusion is important in terms of dietary intake. A positive relationship for the coefficient of determination was calculated ( $R^2 = 0.841$ ), where higher fluoride concentration in a tea infusion correlates to the higher concentration in the original tea product. Therefore, similar to the concentration of fluoride in tea products, Pure blends and Oolong/Pu'er teas had the least fluoride in their infusions with Economy teas containing the most. This was in agreement with existing literature studies and indicates that different tea products provide varying levels of dietary fluoride (Fung *et al.* 1999; Shu *et al.* 2003; Malde *et al.* 2006; Koblar *et al.* 2012).

Many of the UK tea products used give recommended infusion times on their packaging and these ranged from 3 - 5 minutes. In this study, products were infused for time periods of 2, 10 and 30 minutes and the findings showed that overall fluoride concentration increased with the length of infusion time. Compared to the 2 minute infusion, a further 14.9% increase in fluoride ( $\text{mg l}^{-1}$ ) leached out after infusing for 10 minutes, increasing to 20.8% after a 30 minute infusion time. Hence, these results indicate that fluoride continues to leach into a tea infusion over time.

The smaller the particle size of the tea product, the faster fluoride was released into the infusion. If tea is milled to  $<125\ \mu\text{m}$ , fluoride release is rapidly increased, even after 2 minutes, agreeing with Gulati *et al.* (1993) where increased leaching of fluoride was observed with decreasing grain size. Oolong and Pu'er tea products have a larger particle size, compared to the other tea groups (Lu *et al.* 2004) which may have accounted for the slow release of fluoride. Tea from the tea bag products were observed to have smaller particle sizes compared to loose leaf teas, especially in the Economy tea blends, which were almost in a powdered form (Appendix 1).

The tea bag idea, known as a 'tea cartridge' was patented in 1920, in the USA, but it was not introduced to the UK until much later (Challoner 2010). Tetley first introduced the tea bag to the UK in 1953 as Britons preferred traditional leaf products (Tetley 2013). However, tea bags are often filled with mature tea leaves and low cost tea with a higher content of elements than the younger leaves (Malinowska *et al.* 2008; Szymczycha-Madeja *et al.* 2012). This is relevant to the findings in this study with the bagged Economy blend teas and enhances the theory of the use of mature leaves in their manufacture. Today, 96% of Britons use tea bags over loose leaf tea (Tea Council 2013c). The convenient use of a tea bag will most likely continue to be favourable compared to loose tea. However, consideration should be made to the smaller particle sized tea within the bag, leading to a rapid fluoride release into the infusion and therefore a risk of human exposure to higher fluoride.

Tea is not the only source of fluoride in the human diet. Other sources include oral health products - artificially added to toothpastes and mouthwash; grape juice and wine - from the use of pesticides; processed meats - from the breakdown of bone particles; and medicines – from the chemical ingredients, Table 7.1 (Pehrsson *et al.* 2000; Connett and Connett 2001; Fein and Cerklewski 2001; Poureslami *et al.* 2008;

Fluoridealert 2013). In the late 1950's Proctor and Gamble (USA) marketed the first fluoridated toothpaste product, clinically proven against reducing dental cavities (Proctor and Gamble 2013). However, it was not until the late 1970's when the majority of toothpaste companies in the UK had followed suit (Connelly 2010). Today it is not easy to find toothpaste without the addition of fluoride in a UK supermarket. The concentration of fluoride in most European toothpastes is approximately 1450 mg l<sup>-1</sup>, whilst in the US, toothpastes have less fluoride, approximately 1100 mg l<sup>-1</sup> (Bryson 2006). Dentists recommend brushing teeth at least twice a day and it is a common practice all over the world. Although dentists advise the toothpaste is not swallowed due to the fluoride ingredient, in most instances a fraction will be ingested, especially in young children (De Almeida *et al.* 2007). Since 1964, the introduction of artificially fluoridated drinking water began in the UK (Gibson-Moore 2009). Today 10% of the UK population receive the 'optimum' mean dose of 1 mg l<sup>-1</sup> from either natural or fluoridated water supplies (British Fluoridation Society 2013b). In Birmingham, UK, Severn Trent Water supply artificially fluoridated water to their customers (British Fluoridation Society 2013a). This study used ultra-pure de-ionised water in the tea infusion preparation; therefore if tea is prepared using fluoridated drinking water there may be a higher risk to human health, by exceeding the DRI of fluoride in the diet.

Table 7.1 Other sources of fluoride in the diet

Dietary source of fluoride	Concentration (mg l <sup>-1</sup> )
Cola carbonated beverages	≤0.78 mg l <sup>-1</sup> (Pehrsson <i>et al.</i> 2000)
Raisins	2.34 mg kg <sup>-1</sup> (Pehrsson <i>et al.</i> 2000)
Wine	≤2.02 mg l <sup>-1</sup> (Pehrsson <i>et al.</i> 2000)
Processed meats (e.g re-formed chicken and ham)	3.60 mg kg <sup>-1</sup> (Fluoridealert 2013)
Mouthwash	≤5000 mg l <sup>-1</sup> (Fluoridealert 2013)
Toothpaste	≤1450 mg l <sup>-1</sup> (Bryson 2006)

## 7.6 Fluoride in tea and health implications

As an essential micro-nutrient, fluoride is required to sustain healthy bones and teeth, in an optimal range from 1 - 2 mgkg<sup>-1</sup> according to MRC (2002) and WHO (2004). Deficiency can lead to health implications such as dental caries, and in the extreme, an excess can cause skeletal fluorosis (Cao *et al.* 1998; Cao *et al.* 2005; Baskaradoss *et al.* 2008). Although, this is generally known amongst scientists, at present UK guidelines have not been set for fluoride dietary intake by humans. The European Food Safety Authority (EFSA) is consulting on future EU guidelines, recommending 0.05 mg per kg of body weight for fluoride in both adults and children (EU Food Law 2013). However, in the USA, the DRI guideline is currently set at 4 mg per day which is recommended for adult males and 3 mg per day for adult females with an upper tolerable intake of 10 mg per day (NAS 2004). For children from 1 - 13 years, the DRI is recommended between 0.7 - 2 mg per day. These guidelines are provided with the Government knowledge that 72 % of people in the USA, receive a primary source of fluoride from their artificially fluoridated water supplies (Zernike 2012).

With tea providing an additional source of fluoride, it was determined that if an adult male consumes 1000 ml of an Economy blend tea daily, after infusing for 2 minutes, 75 - 120% of the DRI of 4 mg would be available in the presence of food (Ophaug 1990). If consuming a different tea variety such as a Pure blend, the DRI is dramatically reduced, providing 25 - 40%. This is in agreement with Koblar *et al.* (2012) who report that the adequate intake of fluoride from a 70 kg adult consuming five cups of tea daily ranges from 25 - 210% depending upon tea brand and whether the water is fluoridated. For women and children, the DRI is set at a lower value and therefore, a higher percentage of fluoride would be available for consumption.

Infusing tea for longer periods of time as recommended by the tea manufacturers (between 3 and 5 minutes) would also increase the percentage DRI.

Fluoride concentration in the infusion establishes its presence in a tea infusion; however, the fluoride available for absorption by the human gut was determined by developing an *in vitro* laboratory approach. *In vivo* studies have been reported for fluoride in tea, using mammals (Trautner and Siebert 1986; Trautner and Einwag 1989; Cerklewski 1992; Rigalli *et al.* 1994; Buzalaf *et al.* 2008; Suarez *et al.* 2008), but an *in vitro* method for fluoride in tea has not been reported. Using protocols similar to PBET, where artificial body fluids are prepared and digestion is simulated, the *in vitro* oral bioaccessibility of fluoride from tea is reported here for the first time (Powell *et al.* 1998; Bermejo *et al.* 2003; Intawongse and Dean 2008).

Results showed that a high percentage of fluoride from a tea infusion can be accessible in the human gut for possible uptake under fasted conditions. This was estimated at 91.4 - 100% in the oral and gastric compartments combined with the potential for further absorption in the small intestine at 92.1 - 100% and was not dependent upon the tea brand. In the acidic conditions of the stomach, fluoride from tea would most likely exist in the ionic form (Horie *et al.* 1992), with a possibility of conversion to hydrofluoric acid which would readily pass through body membranes to enter the blood. This agrees with the rapid 100% absorption of fluoride into blood plasma (of rats) observed by Rigalli *et al.* (2004). With the addition of milk, a reduction of accessible fluoride (73.8 - 100% in the gastric and 83.1 - 100% in the gastro-intestinal compartments) was observed due to the possible coagulation effects of milk (Trautner and Einwag 1989). Milk is commonly added to black tea blends which would reduce the bioaccessible fluoride, although it is not common practice to add milk to green tea blends.

Considering the metabolism of fluoride, it is reported from *in vivo* studies that fluoride is rapidly removed from the blood plasma via excretion or taken up into the skeletal calcified tissues (Whitford 1996; Cerklewski 1997; Simpson *et al.* 2001; Buzalef *et al.* 2008; Suarez *et al.* 2008; Buzalaf and Whitford 2011). This would account for the health implications associated with excess fluoride in the diet as the teeth and skeleton are affected by fluorosis.

Tea accounts for a high percentage of daily fluoride intake in the diet (Ophaug, 1990). In certain tea commodities, fluoride concentrations in the infusions can exceed the recommended DRI of 4 mg a day (NAS 2004). If consuming tea as the first drink of the day before consuming food in the fasted state, a higher percentage can be absorbed by the human system (Ophaug 1990). Daily fluoride exposure can be increased by considering other sources of fluoride, especially oral hygiene products. Processed food and drinks made using fluoridated water and seafood are other main fluoride sources, Table 7.1 (Chan and Koh 1996; Warren and Levy 1999; Pehrsson *et al.* 2000; Connett and Connett 2001; Fein and Cerklewski 2001; Poureslami *et al.* 2008).

For the consumer, fluoridated water supplies may not be a choice, however, choosing tea type and brand is. Similar to oral hygiene products based on the results of this study, it was felt that the concentration of fluoride should be stated on tea packaging. This will allow the public the choice of a good quality of tea and an indication of how much fluoride they are consuming. This is indeed important to parents who allow their children to drink tea regularly, as the DRI is set much lower up to 0.7 - 1 mg per day for children between the ages of 1 - 8 (NAS 2004). A child could easily exceed these limits from the use of fluoridated toothpaste and from the consumption of fluoridated water alone. Even since introducing fluoridation into the UK, in 2012, 27.9 % of children under five years old were suffering with tooth decay (Public Health England 2013). The greatest levels of tooth decay were reported to be

in the North East and North West of England, where significant deprivation exists in many of the regions, such as Liverpool, Blackburn and Middlesbrough (Department for Communities and Local Government 2011; Innes 2013). If the habitants are economically disadvantaged, it may be that supermarket Economy labels are used and in the case of tea, this would lead to a higher fluoride exposure. With excess fluoride also the cause of dental issues, it should be considered whether these children could be suffering from excess fluoride exposure.



## **Chapter 8: Conclusions and recommendations**

## 8.1 Introduction

The overall aim of this project was to investigate the concentrations of fluoride in tea *Camellia sinensis* (L.) with special reference to human bioaccessibility studies and to consider possible health related issues. Towards achieving this, specific objectives were related to each chapter.

## 8.2 Overall thesis conclusions

The objective for the first experimental chapter (Chapter 2) was, 'to develop analytical methods to determine total fluoride in tea products, infusions, plants and soil.' For total fluoride concentration in tea products, plants and soils, several digestion methods were attempted, the most favoured was a modified version of Sparks *et al.* (1996) and Metrohm (2008), which produced homogenous sample fusion. IC gave a good separation of the fluoride peak from the other anions was achieved with CRM recoveries from 95 - 100%. This methodology was adopted for use in Chapter 3, 'to determine fluoride concentration in the tea products,' and Chapter 6, 'to determine fluoride concentrations in plant materials and soils.'

For fluoride concentration in tea infusions, timed intervals of 2, 10 and 30 minutes were chosen to reflect typical and extreme brewing times (Duckworth and Duckworth 1978; Fung *et al.* 1999; Shu *et al.* 2003; Teablog 2010). Due to the organic nature of tea, IC detection was not feasible; therefore, ISE was adopted as the detection method. Fluoride recovery of the CRM ranged from 93 - 114%. This methodology was adopted in Chapter 4, 'to determine fluoride in tea infusions' and in Chapter 5, 'to determine fluoride concentrations in the gastric and gastrointestinal compartments of human digestion.'

The objective for Chapter 3 was, 'to determine total fluoride in a range of mainly UK available tea products.' Thirty eight tea products were selected as representative of the UK tea market. The teas were classified into groups labelled as, Pure blends, Oolong/Pu'er, Black blends, Green blends and Economy blends. Overall total fluoride in the samples ranged from 103 – 839 mgkg<sup>-1</sup>, reflecting a significant variability ( $p < 0.001$ ). From the groups, Pure blends had the lowest fluoride concentrations and the Economy blends were in the upper range. Economy blends compared with fluoride concentrations of Brick teas, which are known to contain mature leaves in their manufacture (Fung *et al.* 1999; Shu *et al.* 2003).

The Chapter 4 objective was, 'to determine fluoride concentration in tea infusions prepared from the tea products, using a range of infusion times.' From the thirty eight tea products, fluoride concentrations ranged from 0.4 – 8.0 mg l<sup>-1</sup> for a 2 minute infusion times, with an overall mean concentration of  $3.9 \pm 2.2$  mg l<sup>-1</sup>. In assessing human exposure, the adult male DRI for fluoride is recommended as 4 mg/day (NAS 2004). Using the overall mean fluoride concentration of 3.9 mg l<sup>-1</sup> (2 minute infusion), analysed in Chapter 4, consuming one litre of tea would almost fulfil the DRI. However, it does depend upon the tea brand and the quantity of tea consumed, for example, if an adult consumes one litre of tea daily using an UK supermarket Economy brand, the DRI can then be exceeded, which could lead to detrimental health effects. However, exposure to fluoride would be reduced if consuming a Pure blend or Oolong/Pu'er type of tea. In addition, percentage elemental fluoride transfer increased with infusion time. Although it may not be common practice to brew for 10 or 30 minutes, the use of a teapot with loose tea leaves could increase the overall infusion period, therefore increasing the fluoride concentration of the tea beverage.

Chapter 5 addresses the objective, 'to develop an *in vitro* method to estimate the human bioaccessibility of fluoride from tea consumption.' This estimated how much of the fluoride in the tea infusion can be available for absorption during digestion. A laboratory method was developed using artificial saliva and gastric juice, which mimicked human digestion by re-creating typical digestion transition times, movement and conditions. Bioaccessibility of fluoride in tea infusions was estimated to be in the range of 91.4 - 100% in the gastric compartment of human digestion, with further fluoride available for absorption in the small intestine (gastrointestinal fraction), ranging from 92.1 - 100%. Although, the actual concentration of fluoride to which a human is exposed to will be dependent upon the choice of tea type. Pure blend, Twinings Assam would provide  $1.3 \text{ mg l}^{-1}$  of fluoride after gastrointestinal digestion, whereas Economy blend, Morrisons Value bags 2 can deliver over four fold of available fluoride,  $6.0 \text{ mg l}^{-1}$ , compared to the Pure blend.

The uptake of fluoride from soil by the tea plant (*C. sinensis*) and its influence on the plants growth was assessed in Chapter 6. Fluoride doses in excess of  $50 \text{ mg kg}^{-1}$  lead to *C. sinensis* favoured accumulation mainly in the mature leaves of the plants. All plants dosed with fluoride showed signs of marginal necrosis, although the plants continued to grow for the experimental period. The growth rate was significantly slower in the fluoride treated plants as compared with the control plants. An increased accumulation of fluoride in the root systems was observed to occur which was possibly due to the breakdown of the plants natural exclusion mechanism of fluoride.

Even at 'normal' background fluoride concentrations, *C. sinensis* accumulated fluoride into the leaves. With the leaves of *C. sinensis* being 100% of the raw material for the production of tea products, hence all tea products from *C. sinensis* will contain a certain concentration of fluoride. Therefore, human exposure to fluoride from tea consumption is inevitable. With fluoride bioaccessibility being over 90% in

the gastric compartment of human digestion, a fraction or essentially all of this can be absorbed into the human circulatory system. Once in the circulatory system, exposure from the fluoride has occurred and may lead to detrimental health effects such as dental or skeletal fluorosis. However, the daily amount of fluoride ingested from tea could be managed by the choice of brand, limiting the brewing time and the volume that is consumed per day. If fluoride concentrations were stated on food packaging, the level of human exposure could be managed more efficiently at an individual level, hence reducing the possibilities of any fluoride related human health effects.

### **8.3 Future prospects and recommendations**

*C. sinensis* is a relatively slow growing shrub and more plant growth information could be provided if the experiment was conducted over a longer period of time. In this study, the experimental period was limited to 3 months over the summer, due to the UK's cooler climate. The plant normally thrives in warm and humid climates in high altitude locations. Improving the glass-house conditions such as regulating the temperature and humidity to support an extended experimental period would allow the plants to mature further and would provide greater sample mass.

Fluoride concentrations in the tea products and infusions varied significantly ( $p < 0.001$ ) depending upon tea type and brand. Representing the UK tea market was difficult because of the number of varieties and brands available to the consumer. More samples were purchased and analysed than originally planned, after initial findings, but eventually a limit had to be established for this study. With fluoride concentration being so variable, all tea brands and varieties should be analysed, and also an assessment of batch variability, particularly in the case of blended tea. This could be used to build a nutritional data-base for the manufacturers and to raise public awareness.

Mimicking the *in vitro* digestive system in this study was developed using a basic approach. This provided an estimation of oral bioaccessibility of fluoride from tea. The model included the oral, gastric and intestinal compartments, but did not include the large intestine where further fluoride could possibly be accessible. As an addition to the present study, the large intestinal compartment could be introduced. Water absorption is the main function here and any fluoride remaining could be re-absorbed (Pocock *et al.* 2013). Adding the large intestine would involve mimicking a typical transition time and replicating the resident bacterial environment (Smith and Morton 2010).

At present CRM's do not exist for bioaccessibility tests, which can limit the validation of any experimental work. The main reason is the different methodologies that exist to mimic human digestion, Chapter 5 (Lock and Bender 1980, Crews *et al.* 1983, Ruby *et al.* 1996, Williams *et al.* 1998, Rodriguez *et al.* 1999, Smith *et al.* 2000, Lopez *et al.* 2002, Oomen *et al.* 2003, Saikat 2004; Dean and Ma 2007). Variations include differences in intestinal juice composition, digestion times and standardised equipment (Kock *et al.* 2013). Difficulties in standardising these methods were highlighted by Kock *et al.* (2013), where fourteen laboratories took part in a validation test. The laboratories chose to use their own methodologies, described as either complex or simple. Results between laboratories showed variation, for example, accessible lead ranged from 45 - 83%; and zinc from 18 - 56% (Koch *et al.* 2013). The protocol validation test highlighted the need to minimise the variation in methodology, such as pH, incubation times, and juice compositions. At present, the only way to completely validate these *in vitro* tests is to compare against *in vivo* animal models. Therefore, standardising a method, to include reagents and equipment, and producing CRM's are areas for future development.

With tea being the second most popular beverage in the world, public demand will continue, therefore an increased knowledge concerning fluoride in tea is required. Tea breeding and cloning programmes currently focus on producing plants with optimum yields, flavour quality and tolerance to pests and disease (Mondal 2009; Gonbad *et al.* 2013; Mphangwe *et al.* 2013). Further studies need to focus on understanding fluoride uptake and distribution within *C. sinensis*. This could also include identifying possible ways to reduce the fluoride accumulation, so less could be present in the tea product. Further research could include:

- Do different cultivars of *C. sinensis* respond differently to fluoride in terms of tolerance and compartmentation?
- Are 'land races' tea cultivars more tolerant to fluoride than modern commercial varieties?
- What are the variations in fluoride uptake and distribution in *C. sinensis* between countries of origin and the location of the tea plantations (environmental and climatic factors)?
- Do fluoride concentrations in the plant vary over seasons and how does this relate to harvest time?
- Would a reduction of fluoride in the tea plant affect the flavour of the tea product?
- Does the distribution of particle size characteristics of a tea product make any significant differences to the results?

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## **APPENDICES**

## Appendix 1

### Photographs of selected tea products highlighting varying particle size



2 cm

1. PG Tips leaf
2. PG Tips bags
3. Jacksons bags
4. Clipper green leaf
5. Tetley green bags
6. Yunnan Pu'er leaf
7. Fujian Oolong
8. Asda Smart price bags
9. Morrisons Value bags
10. Tesco Value bags
11. Sainsbury Basics bags

## Appendix 2 - Publications produced from the PhD work

### Conference presentations:

Mehra A., Lynch P.T., Saikat S. Q., **Chan L.**, Samson L. and Squires F. (2012). Human exposure to fluoride in Derbyshire, UK. 9<sup>th</sup> International Symposium on Environmental Geochemistry. July, 2012, Aveiro, Portugal.

**Chan L.**, Mehra A., Lynch P. and Saikat S. (2011). Potential human exposure to aluminium and fluoride from tea (*Camellia sinensis*) consumption, at the International Conference on Environment and Health, incorporating the 28th SEGHS (Society for Environmental Geochemistry and Health) European Conference and Workshops, April 2011, Edge Hill, Ormskirk, UK.

**Chan L.**, Mehra A., Lynch P. and Saikat S. (2011). Towards development of an *in vitro* method to estimate the oral bioavailability/bioaccessibility of fluoride from tea consumption at the International Conference on Environment and Health, incorporating the 28th SEGHS (Society for Environmental Geochemistry and Health) European Conference and Workshops, April 2011, Edge Hill, Ormskirk, UK

**Chan L.**, Mehra A., Lynch P. and Saikat S. (2010). Human Exposure to fluoride from tea (*Camellia sinensis*). At the SEGHS 2010 International Conference and Workshops of the Society for Environmental Geochemistry and Health on Environmental Quality and Human Health, June-July, 2010, Galway, Ireland.

### Journal paper:

**Chan L.**, Mehra A., Saikat S. Q. and Lynch P. T. (2013). Human exposure assessment of fluoride from tea (*Camellia sinensis* L.): A UK based issue? *Food Research International*. **51** 564-570.

### Chapter in edited book:

Mehra A., Lynch P., Saikat S., **Chan L.** (2013). Trace elements in tea (*Camellia sinensis*) and their bioavailability. In: *Tea in Health and Disease Prevention*. Preedy V. (Ed.), London: Elsevier. pp. 257-274.

### Appendix 3 - Copy of the journal paper (pre-print)

**Removed for copyright reasons**

Journal paper reference:

Chan L., Mehra A., Saikat S. and Lynch P. (2013). Human exposure assessment of fluoride from tea (*Camellia sinensis* L.): A UK based issue? Food Research International. **51** 564-570.